PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
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Serial No.: 08/918,407

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For: METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND p53

Group Art Unit: 1636

Examiner: W. Sandals

Atty. Dkt. No.: INRP:050/HYL

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37 C.F.R. §1.8

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APPEAL BRIEF (AMENDED TWICE)

BOX AF
Hon. Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief (Twice Amended) in response to the Notification of Non-Compliance With the Requirements of 37 C.F.R. § 1.192(c) dated January 17, 2001. This brief is due on February 17, 2001, based on the mailing date of the Notification of Non-Compliance. The fee for filing this Appeal Brief was submitted on October 24, 2000 with the original Appeal Brief. Should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the

appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/10012461/1973. Please date stamp and return the enclosed postcard to evidence receipt of this document.

I. REAL PARTY IN INTEREST

The real parties in interest are the assignee, the Board of Regents, University of Texas System, Austin, TX, and the licensee, Introgen Therapeutics, Austin, TX.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases. All related cases have issued as U.S. patents (5,747,469 and 6,069,134).

III. STATUS OF THE CLAIMS (AMENDED)

Claims 1-45 were filed with the original application. Claims 46-135 were added during prosecution. Claims 21, 27-31, 62-76, 80-82, 92-95, 102-110, 113, 114, 121-126 and 131-135 were canceled. In the final Office Action Claims 1-26, 32-61, 77-79, 83-89, 96-101, 111, 112, 115-120, and 127-130 were rejected. In the Advisory Action dated June 21, 2000, claims 1-20, 22-26, 32-61, 77-79, 83-91, 96-101, 111, 112, 115, and 127-130.[1]

Claim 127 is cancelled in the Amendment accompanying this Appeal Brief, and claims 2, 5, 33, and 98 were amended to address typographical errors and inconsistencies within the claims. Thus, claims 1-20, 22-26, 32-61, 77-79, 83-91, 96-101, 111, 112, 115-120, and 128-130 are pending and stand appealed. A copy of the appealed claims, with the present amendments indicated, is attached as APPENDIX 1 to this brief.

[1] Claims 116-120 were not identified as rejected in the Advisory Action but were rejected in the Final Office Action. Appellants assume that claims 116-120 remain rejected since an allowance of these claims was not indicated in the Advisory Action.

IV. STATUS OF AMENDMENTS

Appellants are filing an amendment, concurrent with this brief, to address a minor inconsistency within the claims and typographical errors. Exhibit A.

V. SUMMARY OF THE INVENTION

The present invention is directed to therapeutic methods of reducing the growth rate in tumors involving contacting a cell within the tumor with (a) a gene encoding a functional p53 protein and (b) a DNA damaging agent, in a combined amount that is effective to inhibit the growth of the tumor (claim 1). The present invention also concerns compositions that include a gene encoding a functional p53 polypeptide in combination with a DNA damaging agent (claim 32). The invention further concerns therapeutic kits containing a pharmaceutical formulation of a recombinant vector that expresses a functional p53 protein in an animal cell and a pharmaceutical formulation of a DNA damaging agent (claim 42). Specification, *e.g.*, page 7, lines 3-29; page 18, line 32-page 19, line 20.

VI. ISSUES ON APPEAL

- Are the terminal disclaimers submitted herewith sufficient to overcome the statutory double patenting rejection of the present claims based on U.S. Patent 5,747,469?

- Are the present claims obvious over the combined disclosures of Lowe *et al.* ("Lowe"; Exhibit B) or Clarke *et al.* ("Clarke"; Exhibit C), in view of Tishler *et al.* ("Tishler"; Exhibit D), Wills *et al.* ("Wills"; Exhibit E) and Gregory *et al.* ("Gregory"; Exhibit F)?

VII. GROUPING OF THE CLAIMS

For the purposes of the present appeal only, the independent claims, and their respective dependent claims, do not stand or fall together. The independent claims are directed to a method (claim 1), a composition (claim 32), and a kit (claim 42), each with different limitations. The method claims recite "contacting a cell within said tumor with (a) a gene encoding a functional p53 protein and (b) a DNA damaging agent *in a combined amount effective to inhibit the growth of said tumor*," which is not a limitation recited in the other independent claims. The composition claims require "a composition," which is not recited in independent claims 1 or 42. The kit claims recite a "suitable container means," which is not found in the other non-kit claims. The independent claims will therefore stand or fall separately from one another, and separate arguments are presented herewith.

By this action, Appellants are in no way suggesting that other claims do not define patentably distinct inventions. Nevertheless, Appellants believe that the present case presents such clear cut issues of patentability with respect to the independent claims, and Appellants choose to focus on the independent claims in the present appeal.

VIII. ARGUMENT

A. Terminal Disclaimers Overcome Double Patenting Rejection

The pending claims were rejected under the judicially-created doctrine of obviousness-type double-patenting as being unpatentable over claims 1-105 of U.S. Patent 5,747,469. Applicants enclose a terminal disclaimer over the '469 patent, as well as a second terminal disclaimer over U.S. Patent No. 6,069,134. Reconsideration and withdrawal of the rejection is respectfully requested.

B. Claims Are Patentable under 35 U.S.C. §103

The Examiner has rejected all of the claims under 35 U.S.C. § 103 (a) as being obvious over Lowe or Clarke, in view of Tishler, Wills, and Gregory. According to the Examiner, Lowe and Clarke demonstrate that DNA damage caused by radiation induces apoptosis in a p53-dependent fashion. Further, Tishler is said to set forth a long list of DNA damaging agents, and Wills and Gregory are said to support the use of p53 in gene therapy generally. Appellants respectfully traverse.

The invention of claim 1 is concerned with tumor therapy that is achieved by reducing the growth rate of a tumor by contacting cells of the tumor with gene encoding a functional p53 protein and also treating them with conventional chemotherapy (DNA damaging agents). The other independent claims, claims 32 and 42, concern combination compositions and kits.

Appellants will demonstrate that the Examiner failed to make a *prima face* case of obviousness, in that none of the cited references provides the requisite suggestion to produce the subject matter of the claims. The primary references, Lowe and Clark, relate only to the administration of DNA damaging agents to normal (non-tumorous) mouse cells. These references say nothing about gene therapy, say nothing about tumor therapy and say nothing about slowing the growth rate of tumors. Tishler is no more relevant, it also being limited to "mechanistic" studies involving normal mouse cells.

Conversely, the secondary references, Wills and Gregory, concern, at best, only p53 therapy and say nothing and suggest nothing about the desirability of combining p53 therapy with conventional chemotherapy. There is no basis for combining references that concern human therapy with references that concern laboratory observations in rats and mice. In short, the Examiner has merely picked out two elements of the invention, p53 therapy and conventional chemotherapy, from distinct prior art references and failed to demonstrate an appropriate basis

for the proposed combination. The Examiner has failed to properly consider the explicit teachings of these and other references that provide strong evidence of teaching away, the lack of an expectation of success, and indeed, compel a conclusion of non-obviousness.

1. Summary of Cited References

Lowe (Exhibit B) is in no way concerned with gene therapy or even therapy at all. Rather, the authors were trying to understand the various cellular pathways of apoptosis (programmed cell death) that occur in normal mouse cells. Their conclusion was that the intact, normal p53 gene found in normal cells was required for radiation to induce apoptosis in these normal cells. Lowe is merely a scientific investigation paper that concerns the authors' observation that mouse thymocytes having a functional p53 are susceptible to cell death ("apoptosis") when exposed to radiation, while those that do not have a functional p53 gene are more resistant to radiation-induced apoptosis. The authors conclude that apoptosis in the mouse thymus had at least two distinct pathways—a pathway that requires a functional p53 gene and a separate pathway that does not.

It is also notable that radiation is not being used by Lowe to kill or slow the growth of cancer cells. Rather, it is used solely as a convenient laboratory method for inducing apoptosis in thymocytes—a laboratory method that permits the authors to investigate the role of the normal p53 gene in promoting apoptosis in normal mouse thymocytes. Lowe merely attempts to explain why tumors come about in the first place and attempts to explain why the normal p53 gene in the normal cell acts to suppress tumor formation in that normal cell:

> The data presented here define another mechanism by which p53 can act as a tumor suppressor gene [in normal mouse thymocytes]. It has been proposed that the mutational inactivation of p53 during tumorigenesis might allow the further accumulation of oncogenic mutation, due to the removal of an important G1 checkpoint. In thymocytes, and perhaps in other cell types as well, the absence of p53 function can lead to inappropriate cell survival after radiation. The failure to eliminate cells that have

incurred DNA damage could lead to the selection of cells that have undergone neoplastic transformation.

Exhibit B at 848, bottom of col. 2.

Clark (Exhibit C) is a very similar reference to Lowe. Clark is also limited to normal mouse thymocytes cells that have a normal intact p53 gene, and does not in any way concern cells in a tumor that are treated by gene therapy. Clark does not mention gene therapy, much less gene therapy with p53 or gene therapy with p53 and a DNA damaging agent. Based on studies involving compounds that induce apoptosis in normal thymocytes, Clarke simply determined that normal thymocytes lacking p53 are resistant to induction induced by radiation or etoposide. Clark, again, is just a scientific investigation paper that concludes that there are two different apoptosis pathways in mouse thymocytes -- a p53 dependent pathway and a p53 independent pathway. Such a finding may be, and probably was, interesting to scientists at the time, but, like Lowe, it is of no relevance to the presently claimed invention.

The Tishler reference (Exhibit D) is, yet again, another scientific reference that is, frankly, irrelevant to the patentability of the claimed invention. The reference concerns scientific "mechanistic" studies in normal mouse fibroblast cells. It does not discuss tumor cells, or therapy of tumor cells. It does not discuss gene therapy with a man-made p53 gene construct. It does not discuss gene therapy with p53 and DNA damaging agents. This reference merely addresses the following question: what effect do chemotherapeutics and DNA damaging agents have on the DNA-binding function of normal (wild-type) p53 that is already expressed in normal cells? Again, while the answer to this question may well have been interesting to scientists at the time, it is totally irrelevant to the claimed invention. In fact, the experiments in Tishler merely show that when normal mouse fibroblasts cells were exposed to a DNA damaging agent, the

level of the normal cellular p53 protein in these cells increased. The relationship between this teaching and the claimed invention is imaginative at best.

The secondary reference by Wills (Exhibit E) is an abstract that provides little detail. It appears to concern p53 gene therapy but in no way teaches or suggests combination therapy with a DNA damaging agent. Wills purportedly relates to an adenovirus vector that directs the expression of p53 from the Ad 2 major late promoter or the CMV promoter. However, nowhere does Wills mention combining p53 gene therapy with anything else—with any other type of therapy, particularly not therapy with DNA damaging agents or any other type of conventional chemotherapy or radiotherapy.

Like the Wills reference, Gregory (Exhibit F) is a secondary reference that does not mention combining p53 gene therapy with other cancer therapies. It is also an abstract simply discussing adenovirus p53 vectors. It reports that these p53-expressing viruses can suppress DNA replication, inhibit cell growth, and induce apoptosis in certain cell lines.

2. The Cited References Do Not Create a Prima Facie Case

The Examiner argues that Lowe or Clarke, combined with Tishler, Wills and Gregory, render obvious the presently claimed invention. However, this argument is deficient for several reasons. Appellants point to *In re O'Farrell*, 7 USPQ2d 1673, 1680 (Fed. Cir. 1988), in which the Federal Circuit held that a *prima facie* case of obviousness requires that the cited references contain:

(1) detailed enabling methodology for practicing the claimed invention;
(2) a suggestion for modifying the prior art to practice the claimed invention; and
(3) evidence suggesting that the invention would be successful.

It is submitted that the present references relied upon by the Examiner clearly fail to satisfy the tripartite *O'Farrell* test.

a. Cited References Fail to Suggest Their Combination

First, there is no suggestion in any of the cited references to use p53 gene therapy either alone or in combination with a DNA damaging agent to induce apoptosis in cells. While Wills and Gregory may address p53 gene delivery using viral vectors, there is no mention in them either generally or specifically about existing cancer therapies, such as a DNA damaging agent. Nor is there any mention, much less suggestion, to combine them with Lowe, Clarke, or Tishler, which do not address gene transfer in any form, to arrive at the present invention. "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." MPEP § 2143.01 citing *In re Mills,* 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990).

As discussed above, the references of Lowe and Clarke involve administering compounds to **normal mouse** cells to induce apoptosis as a means of studying apoptosis pathways. They compare levels of apoptosis in **normal** cells having endogenous p53 to **normal** cells lacking endogenous p53. Tishler involves the administration of DNA damaging agents to **normal** cells as well and was concerned about their effects on a cell's endogenous p53 level and DNA binding activity. Lowe, Clarke, and Tishler are simply not concerned about the replacement of p53 because they were dealing with **normal**, non-tumor cells. Accordingly, not only is there no suggestion in these three references of p53 gene therapy, but also there is no suggestion that p53 gene therapy should be combined with a DNA damaging agent to constitute tumor therapy. Thus, none of the cited references suggests combining p53 gene therapy with DNA damaging agents or with combining their disclosures to produce the claimed invention.

Appellants further submit that it is incumbent upon the Examiner to find the suggestion to modify the primary reference ***in the prior art***. *In re Soli*, 187 USPQ 797 (CCPA 1963).[2] This has not been done.

The Examiner has argued only that the cited references' authors were "investigating the effects of p53 on cell death." This sort of generalized statement, if true, falls far short of a specific suggestion to modify the primary references—Lowe and Clarke—to introduce a p53 gene into a cell of a tumor. There is no rationale provided as to why one would seek to introduce a p53 gene into a cell when the cell already expresses wild-type p53. More importantly, the references themselves are silent as to why one would seek to change the scientific design described in Lowe and Clarke, especially when their results were "positive" for apoptosis.

This omission from the art is fatal to the Examiner's position, and the attempts to "fill in the blank" with general and unsupported statements constitute an improper hindsight reconstruction of the invention, which is forbidden. *In re Carroll*, 202 USPQ 571 (CCPA 1979) ("One of the more difficult aspects of resolving questions of non-obviousness is the necessity 'to guard against slipping into the use of hindsight.'") (citing *Graham v. John Deere Co.*, 148 USPQ 459 (S. Ct. 1965)). The desirability of the combination of references, as is required by the Federal Circuit, its predecessor court, and the MPEP is simply not provided by the art; therefore, the second prong of the *Farrell* test is unsatisfied, indicating an improper *prima facie* rejection based on obviousness.

[2] "When, as in the instant case, the Patent Office finds, in the words of 35 USC §103, 'differences between the subject matter sought to be patented and the prior art,' it may not, without some basis in logic or scientific principle, merely alleged that such differences are either obvious or of no patentable significance and thereby force an [applicant] to prove conclusively that it is wrong." *In re Soli*, 187 USPQ at 801.

b. There Is No Evidence Invention Would Be Successful

Second, the Examiner has completely glossed over the issue of whether there was an expectation that the claimed invention could be practiced successfully.[3] As has been noted in the previous response, there was no *a priori* predictability, *circa* 1994, with respect to combining ectopic p53 expression with DNA damaging agents. One of skill would have had no way of knowing whether or not cells provided with a vector exogenously expressing p53 expression would give the same results as cells expressing endogenous p53, which were employed in the primary references of Lowe and Clarke.

Also, Lowe and Clarke both involve **normal** mouse thymocytes, not tumor cells. They also both qualify their findings with respect to thymus cells and merely speculate about the applicability of their findings to other cell types. Lowe states, ""These results establish the involvement of p53 in a cell death pathway, specifically radiation-induced apoptosis in the **thymus**." Lowe at 848 (emphasis added). Clarke says, "In conclusion, we have demonstrated a strictly *p53*-dependent pathway to apoptosis in **thymocytes**, cells that are oriented towards programmed deletion by this mode of death." Exhibit C at 850, 852 (emphasis added). Neither paper speculates about the extension of their findings specifically to tumor cells. There is no reasonable expectation of success that their results could, much less should, be extrapolated to tumor cells of the claimed invention.

A statement in the Clarke reference itself implies that there is no reasonable expectation of success in applying their data to other cell types. Clarke distinguishes its findings from those of others in which "*p53* was expressed in a non-physiological environment as part of a

[3] We discuss below the fact that the ordinarily skilled worker at the relevant time frame was unsure as to whether the mechanisms of p53 function were complementary to or, alternatively, counteractive to the mode of action of DNA damaging agents.

recombinant construct and introduced into immortalized cells in which other uncharacterized changes may have occurred. Here, we have studied **inactivation** of one or both copies of the normal gene in otherwise **normal cells**...." Exhibit C at 850. This statement seems to caution that conclusions about non-normal (immortalized) cells and normal cells are not interchangeable because of the differences between the two cell types. Accordingly, Clarke's findings with respect to normal, non-tumor cells may not raise any reasonable expectations with respect to non-normal tumor cells.[4]

Other factors need to be considered when evaluating whether there was a reasonable expectation of achieving the result identified in the application. For example, regulation of the expression of a chromosomal gene, *i.e.*, endogenous p53, is likely to be far different that than observed with a gene provided to a cell by gene therapy, leading to differences in levels of expression. This is correct for a variety of reasons, including, but not limited to, (i) a different promoter (*i.e.*, a non-p53 promoter), (ii) a different chromatin structure surrounding the promoter, transcription start site, or transcription termination site, and (iii) different distal, *cis*-acting regulatory signals not found in exogenous expression constructs. This is especially true when one considers that this very same chromatin may well be damaged by the presence, at least in some embodiments, of DNA damaging agents. Furthermore, there may be temporal differences in expression, such as a reduction of expression some time after a cell is administered gene therapy, which is an observation made in the context of gene therapy vectors that do not integrate into the chromosome, *e.g.*, adenoviral vectors. There simply is no way that the cited

[4] The statement in Clarke also provides support for the argument that this reference does not suggest its combination with Wills or Gregory, which concern tumor cell lines, and not normal cells. This is consistent with the fact that Clarke, like Lowe, is not at all concerned with therapy because it concerns normal cells. Tishler involves normal cells because "studying the DNA-binding function of **wt *p53*** may provide important information on the physiological function of **wt *p53***." Exhibit D at 2212 (emphasis added). Unsurprisingly, no mention, however, is made with regard to implementing a p53/DNA damaging agent treatment.

references, with each of their differences with respect to the claimed invention, present the ***reasonable likelihood of success*** demanded for a *prima facie* case of obviousness.

c. References Do Not Teach All of the Claim Limitations

The Examiner's *prima facie* case is deficient for another reason. "[T]he prior art reference (or references when combined) must teach or suggest all the claim limitations." MPEP § 2142. This combination of references, however, fails to teach limitations expressly recited by the claims.

Claim 1 is directed at a "method of reducing the growth rate of a tumor, comprising contacting a cell within said tumor with (a) a gene encoding a functional p53 protein and (b) a DNA damaging agent in a combined amount effective to inhibit the growth of said tumor." None of the references discuss or suggest providing (a) and (b) "in a combined amount effective to inhibit the growth of said tumor." This is not surprising since none of the references contemplate combined therapy.

Moreover, independent claim 32 recites "a composition comprising a gene encoding a functional p53 polypeptide in combination with a DNA damaging agent. None of the references suggest or teach such a "composition." Even if the combination of cited references taught the administration of gene therapy and a DNA damaging agent, there is nothing to suggest making a composition that included both. Therefore, the "composition" element of the claim has not been taught or suggested by the references.

Independent claim 42 recites a "therapeutic kit, in a suitable container means." Similarly, neither Lowe, Clarke, Tishler, Wills, nor Gregory mentions an element of the claim; in this case "kit," particularly one in a "suitable container means" is not taught or suggested.

The combination of references fails to teach at least one element of each independent claim; consequently, a *prima facie* case of obviousness has not been made on this ground as well.

Thus, for at least three reasons, the Examiner has not carried the burden of establishing a *prima facie* case of obviousness. Therefore, based purely on the deficiencies in the Examiner's analysis, the rejection should be withdrawn.

3. The Art Evidences Conflicting Reports Regarding the Effects of Combining p53 and DNA Damaging Agents

In their previous responses, Appellants have provided an explanation of why, even taking the Examiner's position at face value, the rejection must fail. This explanation was based on the fact that the field was in a state of confusion regarding how p53 and DNA damage interacted in the development of apoptosis at the time of the present invention. This line of argument, presented in detail in the previous responses, was derived from the successful prosecution of the parent of the present application, now U.S. Patent 5,747,469. Appellants present this argument below for the Board's review and consideration.

To underscore the absence of predictability with respect to the instant claims, Appellants point out that the literature, at the time of filing, was in a state of flux as to the potential relationship between p53 and DNA damage. For example, Kastan *et al.* (1991) ("Kastan"; Exhibit G) describes experiments designed to help elucidate the role of p53 in response to DNA damage. As the discussion indicates, DNA damage appears to induce p53, which itself appeared to be associated with an arrest in DNA synthesis. This arrest in DNA synthesis, in turn, was hypothesized to provide the cell with an opportunity to repair any damage to the DNA, preventing transmission of errors in the genetic code to progeny cells.

Taken at face value, this paper clearly raises the question of whether a DNA damaging agent and p53 would work ***against*** each other, given that the point of inducing DNA damage as

part of a cancer therapy regimen is to trigger cell death. By providing an exogenous p53 to treated cells, one would have to consider the possibility that p53 would allow DNA repair to occur, in fact, ***counteracting*** the DNA damage that had been induced, thereby canceling out its therapeutic effect. Tishler, the only reference cited by the Examiner that even addresses this issue, simply confirms Kastan by showing that DNA damage increases p53 levels. ***It does not, however, address the inherent conflict between the cell's desire to repair DNA damage and the clinician's desire to have that damage result in cell death***. This conflict would seem to be exacerbated by the further provision of an exogenous p53 to a tumor cell. Thus, on its face, the teaching of Tishler ***raises*** more questions than it answers.

To further confuse the situation surrounding the interaction between p53 and DNA damage, one must turn to the 1993 paper by Slichenmyer *et al.* ("Slichenmyer"; Exhibit H). This paper reports on the effect of a p53-associated G_1 checkpoint, lost in cells that have defective p53 function, on sensitivity to DNA damage. As stated by the authors, their "results indicate that although the cell cycle checkpoint in G_1 can be impaired through mutation of *p53* or by other mechanisms, [the] loss [of] the G_1 checkpoint *per se* does not influence radiosensitivity or sensitivity to camptothecin." Exhibit H at 4167. Thus, this paper would lead one to the conclusion that the presence of p53 is not a critical factor in the response of a cell to DNA damage, not that p53 could ***cooperate*** with a DNA damaging agent to produce an enhanced therapeutic effect.

Thus, in conclusion, a fair reading of the prior art (including Tishler) could not, as of Appellants' filing date, have provided any reasonable inference, much less a specific suggestion, that a combination of p53 gene therapy and a DNA damaging agent would be a worthwhile

endeavor in the treatment of cancer. Without such an expectation, a case of obviousness cannot exist.

According to the Examiner, as the Slichenmyer and Kastan references were not relied upon, they can "in no way negative the teachings of the later published teachings of Lowe *et al.* and Clarke *et al.*" It is respectfully submitted, however, that the Examiner is mistaken. Slichenmyer, like Lowe and Clarke, was published in 1993, with Kastan published only two years earlier. These reference clearly make up a body of contemporaneous work that cannot be parsed from each other.

In this same vein, it is improper to read the prior art selectively—rather, the prior art must be read as a whole, for all it teaches, as that is what the hypothetical artisan of ordinary skill must do. *See In re Fine*, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988) ("One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention."). Kastan and Slichenmyer constitute the background against which the invention should be considered.

Because these references affirmatively teach ***away*** from the present invention, the case law demands that they must be taken into account and given due weight—not simply discarded when they fail to fit the rejection:

> When prior art contains apparently conflicting references, the Board ***must*** weigh each reference for its power to suggest solutions to an artisan of ordinary skill. The Board ***must*** consider all disclosures of the prior art . . . to the extent that the references are . . . in analogous fields of endeavor and thus would have been considered by a person of ordinary skill in the field of the invention. The Board, in weighing the suggestive power of each reference, ***must*** consider the degree to which one reference might accurately discredit another.

In re Etter, 225 USPQ 1, 6 (Fed. Cir. 1985) (emphasis added). This reasoning gives rise to the doctrines of "failure of others" (*Intel Corp. v. U.S. Int'l Trade Comm'n*, 20 USPQ2d 1161 (Fed.

Cir 1991)) and "teaching away" (*In re Beattie*, 24 USPQ2d 1040 (Fed. Cir. 1997)). If the Examiner were free to ignore contradictory teachings, the "failure of others" and "teaching away" doctrines would have no meaning.

In sum, for the reasons specified, Appellants respectfully submit that the rejection fails to pay sufficient deference to the teachings of the art as a whole. Consequently, it also improperly picks and chooses from the literature and does not address the specific deficiencies of particular references. Reversal of the rejection is, again, requested.

4. At Best, the Rejection Amounts to Impermissible "Obvious to Try" Situation

Appellants strongly argue that the cited references do not suggest the claimed invention and that the art does not suggest a reasonable expectation of success, and in fact, teaches away from the claimed invention. Even if none of these arguments were true, the Examiner's rejection amounts to, at best, an improper "obvious to try" ground for rejection. *See Jones v. Hardy,* 220 USPQ 1021, 1026 (Fed. Cir. 1984). According to *In re Eli Lilly & Co.,* 14 USPQ2d 1741, 1743 (Fed. Cir. 1990), "[a]n 'obvious to try' situation exists when...further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result or indicate that the claimed result would be obtained if certain directions were pursued." Such is the case here. The combination of cited references do not disclose how to achieve a more effective cancer therapy, nor do the references provide even the idea that such a result would be obtained if p53 gene therapy were combined with a DNA damaging agent for the treatment of cancer. Once again, a valid rejection against the claims has not been lodged based on obviousness. The rejection should be withdrawn.

IX. CONCLUSION

It is respectfully submitted, in light of the above, all pending claims are nonobvious under 35 U.S.C. §103. The cited references do not suggest or teach combining references to produce the claimed invention, they do not provide a reasonable expectation of success to produce the claimed invention, the combination of cited references do not teach each limitation of the rejected claims, the art at the time of the invention teaches against the invention, and, at best, the rejection constitutes an improper "obvious to try" situation. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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APPENDIX 1: PENDING CLAIMS (AS AMENDED)

1. A method of reducing the growth rate of a tumor, comprising contacting a cell within said tumor with (a) a gene encoding a functional p53 protein and (b) a DNA damaging agent in a combined amount effective to inhibit the growth of said tumor.

2. (Twice amended) The method of claim 1, wherein said cell is contacted with said gene in combination with X-ray radiation, UV-irradiation, γ-irradiation, microwaves, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C [mytomycin C], or cisplatin.

3. The method of claim 2, wherein said cell is contacted with said gene in combination with cisplatin.

4. The method of claim 1, wherein said cell is contacted with a recombinant vector that expresses a functional p53 protein in said cell in combination with a DNA damaging agent.

5. The method of claim 4, wherein said p53-expressing recombinant, [non-viral] vector is a naked DNA plasmid or a plasmid within a liposome, a retroviral vector, an AAV vector, or a recombinant adenoviral vector.

6. The method of claim 5, wherein said p53-expressing recombinant vector is a recombinant adenoviral vector.

7. The method of claim 4, wherein said p53-expressing recombinant vector comprises a p53 expression region positioned under the control of a constitutive promoter.

8. The method of claim 4, wherein said recombinant vector comprises a p53 expression region, the cytomegalovirus IE promoter and the SV40 early polyadenylation signal.

9. The method of claim 6, wherein at least one gene essential for adenovirus replication is deleted from said adenovirus vector construct and the p53 expression region is introduced in its place.

10. The method of claim 9, wherein the E1A and E1B regions of the adenovirus vector are deleted and the p53 expression region is introduced in their place.

11. The method of claim 6, wherein said recombinant adenoviral vector is present within a recombinant adenovirus.

12. The method of claim 1, wherein said cell is first contacted with said gene and is subsequently contacted with said DNA damaging agent.

13. The method of claim 1, wherein said cell is first contacted with said DNA damaging agent and is subsequently contacted with said gene.

14. The method of claim 1, wherein said cell is simultaneously contacted with said gene and said DNA damaging agent.

15. The method of claim 1, wherein said cell is contacted with a first composition comprising said gene and a second composition comprising said DNA damaging agent.

16. The method of claim 15, wherein said first or second composition is dispersed in a pharmacologically acceptable formulation.

17. The method of claim 1, wherein said cell is contacted with a single composition comprising said gene in combination with said DNA damaging agent.

18. The method of claim 17, wherein said composition is dispersed in a pharmacologically acceptable formulation.

19. The method of claim 17, wherein said cell is contacted with a single composition comprising a recombinant vector that expresses p53 in said cell in combination with said DNA damaging agent.

20. The method of claim 19, wherein said cell is contacted with a single composition comprising a recombinant adenovirus containing a recombinant vector that expresses p53 in said cell in combination with said DNA damaging agent.

21. (Canceled)

22. The method of claim 1, wherein said tumor cell is a malignant cell.

23. The method of claim 22, wherein said malignant cell is a lung cancer cell.

24. The method of claim 22, wherein said malignant cell is a breast cancer cell.

25. The method of claim 22, wherein said malignant cell has a mutation in a p53 gene.

26. The method of claim 1, wherein said tumor cell is located within an animal at a tumor site.

32. A composition comprising a gene encoding a functional p53 polypeptide in combination with a DNA damaging agent.

33. (Amended) The composition of claim 32, comprising said gene in combination with adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D [actimomycin-D], mitomycin C, or cisplatin.

34. The composition of claim 33, comprising said gene in combination with cisplatin.

35. The composition of claim 32, comprising a recombinant vector that expresses a functional p53 protein in an animal cell in combination with a DNA damaging agent.

36. The composition of claim 35, wherein said recombinant vector is a naked DNA plasmid or a plasmid within a liposome.

37. The composition of claim 36, wherein said recombinant vector is a recombinant adenoviral vector.

38. The composition of claim 37, wherein said recombinant vector is a recombinant adenoviral vector is present within a recombinant adenovirus particle.

39. The composition of claim 32, comprising a recombinant adenoviral vector present within a recombinant adenovirus particle in combination with cisplatin.

40. The composition of claim 32, dispersed in a pharmacologically acceptable formulation.

41. The composition of claim 40, formulated for intralesional administration.

42. A therapeutic kit comprising, in suitable container means, a pharmaceutical formulation of a recombinant vector that expresses a functional p53 protein in an animal cell and a pharmaceutical formulation of a DNA damaging agent.

43. The kit of claim 42, wherein said recombinant vector and said DNA damaging agent are present within a single container means.

44. The kit of claim 42, wherein said recombinant vector and said DNA damaging agent are present within distinct container means.

45. The kit of claim 42, comprising a pharmaceutical formulation of a recombinant adenovirus including a recombinant vector that expresses a p53 protein in an animal cell and a pharmaceutical formulation of cisplatin.

46. The method of claim 1, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation, UV-irradiation, γ-irradiation or microwaves.

47. The method of claim 46, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation.

48. The method of claim 46, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with UV-irradiation.

49. The method of claim 46, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with γ-irradiation.

50. The method of claim 46, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with microwaves.

51. The method claim 1, wherein the tumor cell is contacted with a pharmaceutical composition comprising a DNA damaging compound.

52. The method of claim 51, wherein the DNA damaging agent is cisplatin.

53. The method of claim 51, wherein the DNA damaging agent is doxorubicin.

54. The method of claim 51, wherein the DNA damaging agent is etoposide.

55. The method of claim 51, wherein the DNA damaging agent is verapamil.

56. The method of claim 51, wherein the DNA damaging agent is podophyllotoxin.

57. The method of claim 51, wherein the DNA damaging agent is 5-FU.

58. The method of claim 51, wherein the DNA damaging agent is actinomycin-D.

59. The method of claim 51, wherein the DNA damaging agent is adriamycin.

60. The method of claim 51, wherein the DNA damaging agent is camptothecin.

61. The method of claim 51, wherein the DNA damaging agent is mitomycin C.

77. The method of claim 4, wherein said gene is administered prior to said DNA damaging agent.

78. The method of claim 4, wherein said gene is administered after said DNA damaging agent.

79. The method of claim 4, wherein said gene is administered at the same time as said DNA damaging agent.

83. The method of claim 26, wherein said gene is delivered to said tumor endoscopically, intravenously, intratracheally, intralesionally, percutaneously or subcutaneously.

84. The method of claim 26, wherein said tumor site is a resected tumor bed.

85. The method of claim 26, wherein said administration is repeated.

86. The method of claim 13, wherein the period between administration of the DNA damaging agent and gene is between 12 and 24 hours.

87. The method of claim 13, wherein the period between administration of the DNA damaging agent and gene is between 6 and 12 hours.

88. The method of claim 13, wherein the period between administration of the DNA damaging agent and gene is about 12 hours.

89. The method of claim 12, wherein the period between administration of the gene and DNA damaging agent is between 12 and 24 hours.

90. The method of claim 12, wherein the period between administration of the gene and DNA damaging agent is between 6 and 12 hours.

91. The method of claim 12, wherein the period between administration of the gene and DNA damaging agent is about 12 hours.

96. The method of claim 1, wherein said tumor cell is an epithelial tumor cell.

97. The method of claim 23, wherein said lung cancer cell is non-small cell lung carcinoma cell.

98. (Amended) The method of claim 97, wherein said non-small cell lung carcinoma cell is a squamous [sqamous] carcinoma cell.

99. The method of claim 97, wherein said non-small cell lung carcinoma cell is an adenocarcinoma cell.

100. The method of claim 97, wherein said non-small cell lung carcinoma cell is a large-cell undifferentiated carcinoma cell.

101. The method of claim 95, wherein said lung cancer cell is a small cell lung carcinoma cell.

111. The method of claim 26, wherein said gene is administered in about 0.1 ml.

112. The method of claim 26, wherein said gene is administered in about 10 ml.

115. The method of claim 52, wherein said cisplatin is administered at 20 mg/m^2.

116. The method of claim 53, wherein said doxorubicin is administered at 25-75 mg/m^2.

117. The method of claim 54, wherein said etoposide is administered at 35-50 mg/m^2.

118. The method of claim 57, wherein said 5-FU is administered at 3-15 mg/kg.

119. The method of claim 47, wherein the x-ray dosage is between 2000 and 6000 roentgens.

120. The method of claim 47, wherein the x-ray dosage is between 50 and 200 roentgens.

127. (Canceled) The method of claim 4, wherein said promoter is a promoter.

128. The method of claim 7, wherein the promoter is selected from the group consisting of SV40, CMV and RSV.

129. The method of claim 128, wherein the promoter is the CMV IE promoter.

130. The method of claim 129, wherein the vector further comprises a polyadenylation signal.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jack A. ROTH, Toshiyoshi FUJIWARA, Elizabeth A. GRIMM, Tapas MUKHOPADHYAY, Wei-Wei ZHANG And Laurie B. OWEN-SCHAUB

Serial No.: 08/918,407

Filed: August 26, 1997

For: METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND p53

Group Art Unit: 1636

Examiner: W. Sandals

Atty. Dkt. No.: INRP:050/HYL

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

October 24, 2000
DATE

Gina N. Shishima

AMENDMENT UNDER 37 C.F.R. §1.116

BOX AF
Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Appellants respectfully submit the following amendments for entry into the captioned patent application in accordance with 37 C.F.R. § 1.116. This paper is offered in response to the Office Action dated November 22, 1999 and is filed concurrently with an Appeal Brief, submitted herewith. Appellants contend these amendments place the case in even better condition for allowance or appeal as they clarify i) the priority claim in the specification and ii)

the claims by correcting misspellings or eliminating confusing terms. Accordingly, no new matter for which a new search is required has been added.

Reconsideration of the application in view of the following amendments and remarks is respectfully requested.

AMENDMENT

In the Specification

On page 2, line 6, please delete "07/960,543" and insert therefor --07/960,513--.

In the Claims

Please cancel claim 127, without prejudice or disclaimer.

Please amend the claims as follows:

2. (Twice amended) The method of claim 1, wherein said cell is contacted with said gene in combination with X-ray radiation, UV-irradiation, γ-irradiation, microwaves, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C [mytomycin C], or cisplatin.

5. (Twice amended) The method of claim 4, wherein said p53-expressing recombinant[, non-viral] vector is a naked DNA plasmid or a plasmid within a liposome, a retroviral vector, an AAV vector, or a recombinant adenoviral vector.

33. (Twice amended) The composition of claim 32, comprising said gene in combination with adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D [actimomycin-D], mitomycin C, or cisplatin.

98. (Amended) The method of claim 97, wherein said non-small cell lung carcinoma cell is a squamous [sqamous] carcinoma cell.

REMARKS

The amendment to the specification is needed to correct a typographical error in the Specification as filed. Support for this amendment can be found in the inventors' Declaration previously filed with the U.S. Patent Office.

Claim 127 was cancelled because the claim, as previously amended, did not introduce any further limitations than the claim from which it depended. Claims 2, 33, and 98 were amended to correct misspellings. Claim 5 has been amended to clarify an inconsistency in the claim.

Applicants respectfully submit that these amendments does not introduce any new matter into the specification.

The Examiner is invited to contact the undersigned attorney at (512) 418-3081 with any questions, comments or suggestions relating to the referenced patent application.

Please date-stamp and return the enclosed postcard evidencing receipt of these materials.

Respectfully submitted,

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Date: October 24, 2000

p53 is required for radiation-induced apoptosis in mouse thymocytes

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THE *p53* tumour suppressor gene is the most widely mutated gene in human tumorigenesis[1,2]. *p53* encodes a transcriptional activator[3–7] whose targets may include genes that regulate genomic stability[8,9], the cellular response to DNA damage[10,11], and cell-cycle progression[12,13]. Introduction of wild-type *p53* into cell lines that have lost endogenous *p53* function can cause growth arrest[14–16] or induce a process of cell death known as apoptosis[17,18]. During normal development, self-reactive thymocytes undergo negative selection by apoptosis[19], which can also be induced in immature thymocytes by other stimuli, including exposure to glucocorticoids[15] and ionizing radiation[16]. Although normal negative selection involves signalling through the T-cell receptor[14], the induction of apoptosis by other stimuli is poorly understood. We have investigated the requirement for *p53* during apoptosis in mouse thymocytes. We report here that immature thymocytes lacking *p53* die normally when exposed to compounds that may mimic T-cell receptor engagement and to glucocorticoids but are resistant to the lethal effects of ionizing radiation. These results demonstrate that *p53* is required for radiation-induced cell death in the thymus but is not necessary for all forms of apoptosis.

Because of the potential involvement of *p53* in inducing apoptosis[17,18] we studied cell death in thymocytes derived from mice carrying a germ-line disruption of the *p53* gene (T.J. and R. Weinberg, unpublished results). Thymocytes were isolated from *p53* homozygous mutant, heterozygous and wild-type animals and subjected *in vitro* to treatments that induce apoptosis. Although treatment with phorbol ester/calcium ionophore (which may mimic engagement of the T-cell receptor[22]) and dexamethasone induced death with similar kinetics in thymocytes of all three genotypes (Fig. 1*a*, *b*), *p53*-deficient cells displayed a dramatic resistance to the effects of ionizing radiation (Fig. 1*c*). Moreover, the *p53*-deficient thymocytes remained viable following doses up to 2,000 centiGreys (cGy); wild-type cells were susceptible to treatment with as little as 100 cGy (Fig. 2). In addition, at all doses and times examined, cells isolated from heterozygous animals displayed intermediate viability compared to wild-type and homozygous mutant animals (Figs 1*c* and 2).

We further tested the effects of dexamethasone and γ-irradiation on thymocyte survival *in vivo*. Thymocytes isolated from treated animals were examined for the presence of the cell-surface markers CD4 and CD8 using two-colour fluorescence-activated cell sorter (FACS) analysis. Thymuses from untreated normal and mutant animals contained about 75–80% immature, $CD4^+CD8^+$ cells, which are susceptible to apoptosis[23]. Forty-eight hours after treatment with dexamethasone, all thymuses sustained a significant reduction in cell numbers, which could be attributed to selective loss of $CD4^+CD8^+$ cells (Fig. 3*a*, *b*). Similarly, thymuses from wild-type animals exposed to γ-radiation contained a low percentage of $CD4^+CD8^+$ cells (Fig. 3*a*, *b*). In contrast, irradiation of *p53* homozygous mutant animals caused only minor reductions in $CD4^+CD8^+$ cells (Fig. 3*a*, *b*).

Conditions that induced cell death produced the internucleosomal degradation of thymic DNA, which is indicative of apoptosis[24] (Fig. 3*c*). This characteristic 'DNA ladder' was not evident after irradiation of homozygous mutant animals (Fig. 3*c*). Consistent with the data from *in vitro* experiments, mice that were heterozygous for the *p53* mutation were less susceptible than wild-type mice to the effects of ionizing radiation, both in survival of $CD4^+CD8^+$ cells and extent of DNA laddering (Fig. 3*b*, *c*).

Given the apparent requirement for *p53* function in radiation-induced apoptosis of thymocytes, we examined the steady-state level of p53 protein in wild-type cells after exposure to ionizing radiation. Consistent with findings in other cell types[10,11], irradiation of thymocytes caused a dramatic increase in p53 levels. The accumulation of p53 protein was apparent within 1 h, before significant degradation of DNA (Fig. 4 and data not shown).

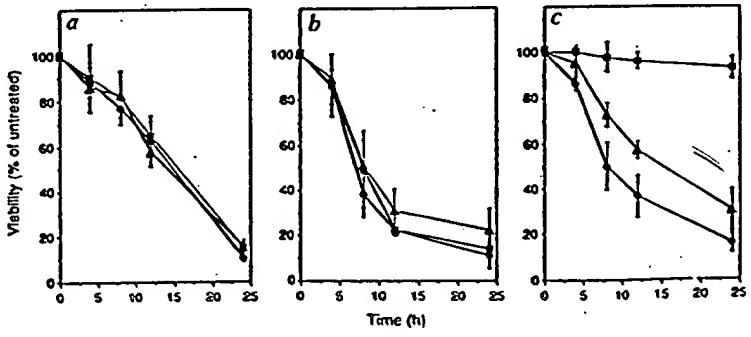

FIG. 1 Induction of apoptosis in isolated thymocytes. Thymocytes were treated with: *a*, 10nM phorbol ester (phorbol 12-myristate 13-acetate, PMA) and 500 nM calcium ionophore (A23187); *b*, 1 μM dexamethasone; or *c*, 500 cGy ionizing radiation and viability was assessed at various times thereafter. Thymocytes were derived from *p53* homozygous mutant (■), heterozygous (▲) and wild-type (●) animals. METHODS. Mutant mice used in these experiments carry a germ-line disruption of the *p53* gene (T.J. and R. Weinberg, unpublished results) that was made by gene targeting in D3 embryonic stem cells[32]. The mutation consists of a replacement by the bacterial *neo* gene of *p53* sequences between exons 2 and 6; immunoprecipitation analysis has confirmed that the mutation eliminates production of p53 protein[6]. The mutation is carried on a hybrid (C57BL/6×129/sv) genetic background. Although genetic background influences the sensitivity of thymocytes to treatments such as irradiation[33], we obtained consistent results from all animals within a given genotype. Thymocytes were isolated from mice age 4.5–7 weeks in tissue culture media (DME supplemented with 5% fetal bovine serum and 25 mM HEPES, pH 7.2) and adjusted to a density of 1×10^6 per ml. At time zero, cultures were treated, divided into 16-mm wells (1×10^6 per well) and incubated at 37 °C. The relative amounts of nonviable cells were determined at various times by uptake of fluorescein isothiocyanate (FITC) and FACS analysis[34,35]. Values represent the average viability from four independent experiments with standard deviations; each experiment compared cells derived from one mutant, one heterozygote and one wild-type animal and were normalized to untreated samples from the same animal. Two experiments used littermates derived from F_1 crosses. There were no significant differences between viability of untreated thymocytes over the period of the experiment (not shown); on average, viability of untreated cells was about 70% at 24 h. Cell death by apoptosis was confirmed by analysis of genomic DNA (data not shown; see Fig. 3). Irradiation was done with a GammaCell 40 equipped with a ^{137}Cs source.

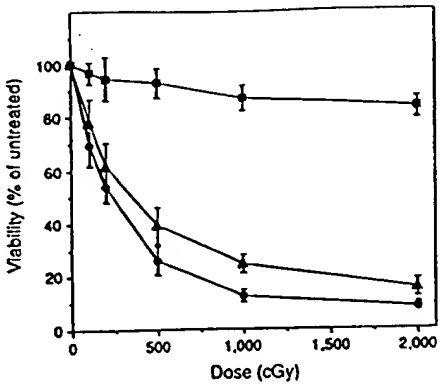

FIG. 2 Viability of isolated thymocytes treated with varying doses of ionizing radiation. Thymocytes were isolated from *p53* homozygous mutant (■), heterozygous (▲) and wild-type (●) animals and treated with varying doses of ionizing radiation. Viability was assessed 20 h after treatment as described in Fig. 1. Values represent averages from three independent experiments and are normalized to the amount of viable cells remaining in untreated cultures derived from the same animal.

In contrast, treatment with phorbol ester/calcium ionophore and dexamethasone resulted in little or no increase in p53 levels.

These results establish the involvement of *p53* in a cell death pathway, specifically radiation-induced apoptosis in the thymus. Equally important, these data demonstrate that apoptosis can also occur in the absence of *p53* function. Thus, cell death in the thymus can be subdivided into at least two distinct pathways, one requiring *p53* and one that is *p53* independent. The existence of multiple apoptotic pathways in the thymus has been suggested from the analysis of *bcl-2* transgenic mice[17,18]. Furthermore, the apparently normal development of mice homozygous for a *p53* mutation (ref. 27 and T.J. and R. Weinberg, unpublished results) suggests that *p53* is not required for cell death in many, perhaps most, instances.

p53 has been implicated in controlling a checkpoint during the G1 phase of the cell cycle that may monitor the state of the DNA before entry into S phase[11,28]. For example, *p53*-deficient fibroblasts fail to arrest transiently in G1 after γ-irradiation, although they still pause normally in G2 (ref. 11). A rapid accumulation of p53 precedes G1 arrest in fibroblasts[10,11] and, as shown here, radiation-induced apoptosis in thymocytes. Thus, the different cellular responses (apoptosis versus G1 arrest) may result from the activation of distinct target genes by p53. Alternatively, activation of the same target genes in the two cell types could have different consequences. The fact that elevated levels of p53 can lead to the initiation of apoptosis is consistent with earlier studies that demonstrated a link between *p53* expression and cell death[22,23], and it is possible that many conditions that lead to an accumulation of p53 could induce apoptosis. Those stimuli which cause apoptosis in thymocytes in the absence of *p53* function may use other transcription factors to activate the same set of 'cell death' genes.

The data presented here define another mechanism by which *p53* can act as a tumour suppressor gene. It has been proposed that the mutational inactivation of *p53* during tumorigenesis might allow the further accumulation of oncogenic mutations, due to the removal of an important G1 checkpoint[11,28]. In thymocytes, and perhaps in other cell types as well, the absence

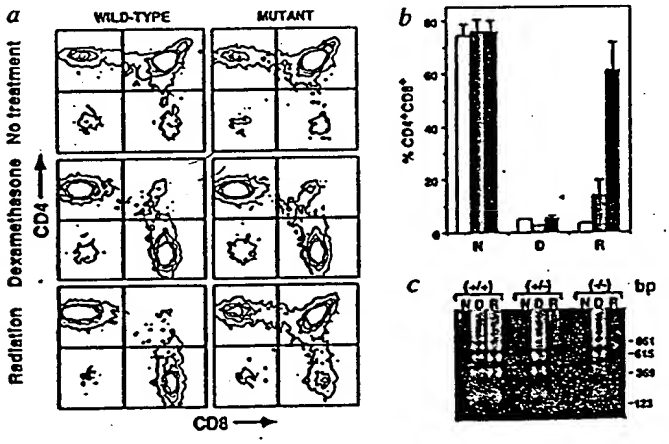

FIG. 3 Thymocyte apoptosis *in vivo*. *p53* homozygous mutant, heterozygous and wild-type animals were treated with dexamethasone or ionizing radiation and isolated thymocytes examined for the cell surface expression of CD4 and CD8 (after 48 h) and the condition of genomic DNA (after 10 h). *a*, Two-colour immunofluorescence contour plots from FACS analysis of CD4 and CD8 surface expression in wild-type and *p53* homozygous mutant mice. *b*, Mean percentage of surviving $CD4^+CD8^+$ thymocytes from wild-type (□), heterozygous (▨), and *p53* homozygous mutant (■) mice 48 h after treatment with dexamethasone (D), γ-irradiation (R), or no treatment (N). *c*, Agarose gel electrophoresis of total thymus DNA from wild-type (+/+), *p53* heterozygous (+/−), and *p53* homozygous mutant (−/−) mice 10 h after treatment with dexamethasone (D), γ-irradiation (R), or no treatment (N). The position of molecular size standards (in base pairs) is shown at right. METHODS. Thymocytes were recovered from mice 48 h after treatment with 0.5 mg dexamethasone (administered by intraperitoneal injection in PBS), γ-irradiation (500 cGy), or no treatment and stained with phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-CD8 antibodies (anti-L3T4 and anti-Lyt 2, Becton Dickinson). Multiparameter analysis of live cells was done on a FACStar Plus (Becton Dickinson). Dead cells were excluded by staining with propidium iodide and by gating of forward and side scatter of light during FACS analysis. The relative contributions of CD4 and CD8-expressing subpopulations were estimated using the Disp2D program (Becton Dickinson). Representative samples are shown in *a*. Values in *b* represent averages from three independent experiments (except the dexamethasone-treated heterozygous samples, which is an average of two experiments). Total genomic DNA was analysed from 10^6 thymocytes isolated 10 h after the treatments described above, according to the protocol in ref. 36.

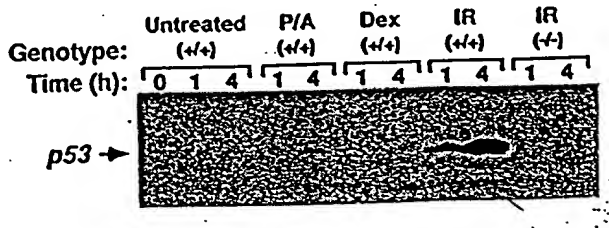

FIG. 4 p53 levels in isolated thymocytes undergoing apoptosis. Thymocytes were isolated from wild-type (+/+) and homozygous mutant (−/−) animals and treated with cytotoxic agents. At 1 and 4 h after treatment, p53 levels were determined by western blot analysis. The treatment and genotype of the cells are indicated above the appropriate lanes: normal tissue culture media (untreated); PMA + A23187 (P/A); dexamethasone (dex); ionizing radiation (IR). The time (in h) after treatment is indicated over each lane. METHODS. Thymocytes were isolated and treated as described in Fig. except the cultures were incubated in 75 cm^2 tissue culture flasks (10 per flask). For each sample, 1×10^7 cells were washed in PBS and lysed Laemmli buffer[37]. The proteins were separated on 7.5% SDS-polyacrylamid gels and transferred to PVDF membranes (Millipore). Membranes were blocked and probed with a pool of p53-specific monoclonal antibodies (PAb421, PAb240 and PAb248)[38,39]. p53 was detected using an alkalin phosphatase-conjugated secondary antibody and a chemiluminescent sub strate (ref. 40; Lumi-Phos 530, Boehringer–Mannheim).

of *p53* function can lead to inappropriate cell survival after γ-irradiation. The failure to eliminate cells that have incurred DNA damage could lead to the selection of cells that have undergone neoplastic transformation. Note that among the various tumour types that occur in *p53* homozygous mutant mice, lymphoma is by far the most common (ref. 27 and T.J. and R. Weinberg, unpublished results), and the four cases of this tumour that have been examined from our *p53*-deficient mice have consisted predominantly of $CD4^+CD8^+$ cells (T.J., unpublished results). Thus, like *bcl-2* activation[29-31], the inactivation of *p53* may contribute to tumorigenesis through an inhibition of apoptosis. □

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Thymocyte apoptosis induced by p53-dependent and independent pathways

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DEATH by apoptosis is characteristic of cells undergoing deletion during embryonic development, T- and B-cell maturation and endocrine-induced atrophy[1]. Apoptosis can be initiated by various agents[1-6] and may be a result of expression of the oncosuppressor gene *p53* (refs 6–8). Here we study the dependence of apoptosis on p53 expression in cells from the thymus cortex. Short-term thymocyte cultures were prepared from mice constitutively heterozygous or homozygous for a deletion in the *p53* gene introduced into the germ line after gene targeting. Wild-type thymocytes readily undergo apoptosis after treatment with ionizing radiation, the glucocorticoid methylprednisolone, or etoposide (an inhibitor of topoisomerase II), or after Ca^{2+}-dependent activation by phorbol ester and a calcium ionophore. In contrast, homozygous null *p53* thymocytes are resistant to induction of apoptosis by radiation or etoposide, but retain normal sensitivity to glucocorticoid and calcium. The time-dependent apoptosis that occurs in untreated cultures is unaffected by *p53* status. Cells heterozygous for *p53* deletion are partially resistant to radiation and etoposide. Our results show that *p53* exerts a significant and dose-dependent effect in the initiation of apoptosis, but only when it is induced by agents that cause DNA-strand breakage.

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of *p53* function can lead to inappropriate cell survival after γ-irradiation. The failure to eliminate cells that have incurred DNA damage could lead to the selection of cells that have undergone neoplastic transformation. Note that among the various tumour types that occur in *p53* homozygous mutant mice, lymphoma is by far the most common (ref. 27 and T.J. and R. Weinberg, unpublished results), and the four cases of this tumour that have been examined from our *p53*-deficient mice have consisted predominantly of $CD4^+CD8^+$ cells (T.J., unpublished results). Thus, like *bcl-2* activation[29-31], the inactivation of *p53* may contribute to tumorigenesis through an inhibition of apoptosis. □

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Thymocyte apoptosis induced by p53-dependent and independent pathways

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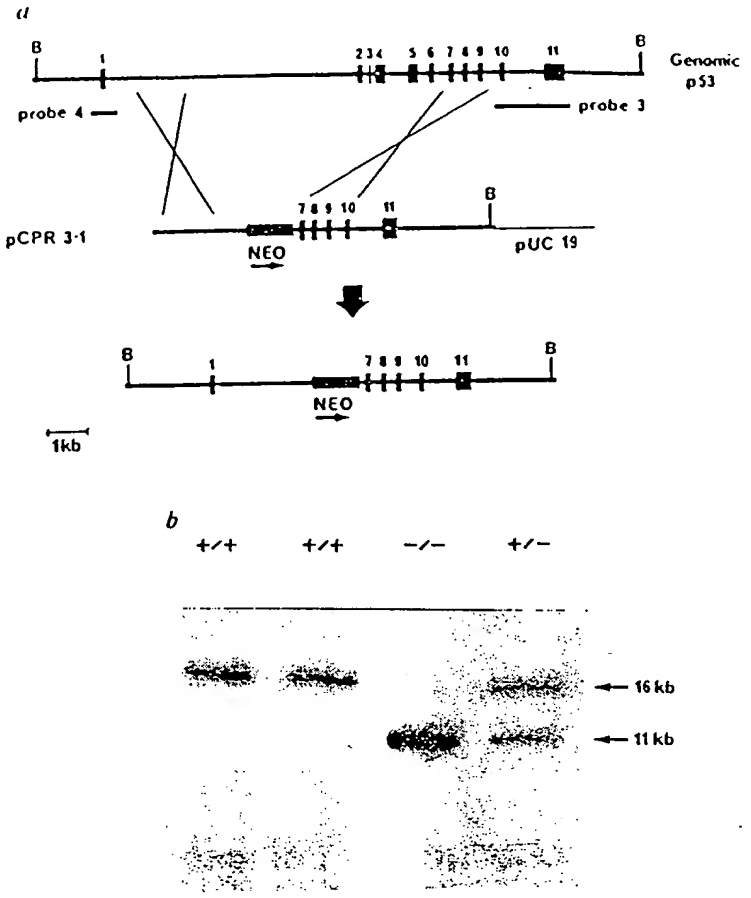

FIG. 1 Strategy for the inactivation of the murine *p53* gene. *a*, The *p53* locus (boxes represent exons 1-11). The vector pCPR3.1 used to generate clone R72 was created by ligation of a *Hin*dIII-*Bam*HI fragment of intron 1 to a *Bam*HI-*Eco*RI fragment encompassing exons 7-11 (from murine genomic *p53* clone pMSVp53G; ref. 22), and then inserting a *pgk-neo* cassette[23] at the junction of these fragments. pCPR5.1 differs by the inclusion of a *pgk-tk* cassette at the *Bgl*II site at the 3′ end of the *p53* homology (1.8 kb *Bgl*II-*Eco*RI HSV-*tk* fragment[24] in the *Sma*I site of a *pgk* expression vector[25]). The two probes used for Southern analysis are indicated, one internal (P3, a 2-kb *Hin*dIII fragment) and one external (P4, a 0.8 kb fragment from a *Eco*RI-*Hin*dIII double digest) to the homology. Thick lines represent genomic DNA; thin lines, plasmid DNA. 'B' indicates a *Bgl*II restriction enzyme digestion site. *b*, Typical Southern analysis of progeny mice after restriction with *Bgl*II and probing with P3. An identical pattern is obtained with P4. The positions of bands corresponding to the wild-type *p53* allele (16 kb) and the *neo*-containing allele (11 kb) are indicated.
METHODS. Electroporation was done as described[26]. After one day, targeted cells were selected in 200 μg ml^{-1} G418 and, when counterselection was required, in 2 μM Ganciclovir in the continued presence of G418 after 4 days. Clones were transferred into G418-containing medium after 10 days. Following identification of targeted clones and production and breeding of germ-line chimaeras, DNA was prepared from tail biopsies, digested, separated by electrophoresis in a 0.8% agarose gel, transferred to a Hybond-N$^+$ filter (Amersham) and hybridized according to the manufacturer's instructions.

homozygous-null thymocytes (Fig. 3*a*); the rates at which these cells entered apoptosis in the absence of an inducing stimulus were comparable (Fig. 3*b*).

Expression of p53 was also investigated immunocytochemically in smears of thymocytes from wild-type mice using the murine-specific antibodies CM-5 (a polyclonal antibody) and PAb242 (a monoclonal antibody against N-terminal epitopes of p53; ref. 10). Over the first four hours of culture, the proportion of etoposide- and radiation-treated cells showing intranuclear p53 rose to 6.5%, whereas less than 3% of nuclei from untreated and glucocorticoid-treated thymocytes were positive.

These data raise several interesting issues regarding the role of the *p53* oncosuppressor gene in apoptosis. Previous studies have shown that expression of wild-type *p53* (but not of the mutated, oncogenic form) induces apoptosis in cells of erythroid[6], myeloid[7] and colorectal epithelial lineages[8]. However, in these experiments *p53* was expressed in a non-physiological environment as part of a recombinant construct and introduced into immortalized cells in which other uncharacterized changes may have occurred. Here, we have studied inactivation of one or both copies of the normal gene in otherwise normal cells from a fully differentiated organ. We show that *p53* has a limiting, gene-dose-dependent effect on apoptosis following some, but not all, types of lethal stimuli. By contrast, using thymocytes from animals heterozygous for an *Rb-1* null allele[11], we found no evidence for a similar role for the retinoblastoma oncosuppressor gene (data not shown).

Ionizing radiation and etoposide both differ from glucocorticoid in causing immediate DNA-strand breaks in thymocytes[12], which in the case of ionizing radiation are mainly single-strand nicks that are repaired within an hour[13]. The irradiated cells are nonetheless committed to apoptosis, which is initiated in a stochastic fashion over the ensuing several hours, suggesting that a lethal signal is generated within the damaged cells that is not revoked by subsequent DNA repair. Intranuclear p53 protein is known to accumulate following DNA damage[14,15], and our data provide evidence that this is part of the lethal signal.

Etoposide stabilizes the topoisomerase-DNA complex during its cleavage-religation cycle, and so generates double-strand DNA breaks, even in non-replicating cells[16]. The reason for the efficacy of such drugs in cancer chemotherapy is not clear, although they can initiate apoptosis in a variety of cell types[12,17,18]. Our data show that the apoptosis caused by etoposide in cortical thymocytes, which are rich in topoisomerase II, depends largely upon *p53*, although additional modes of drug action may apply at higher concentrations.

There must also be a p53-independent pathway responsible for the apoptosis initiated by glucocorticoid treatment, calcium-dependent activation and *in vitro* ageing. The combination of calcium ionophore and the protein kinase C activator, PMA, reproduces many features of the 'activation-induced' death of rodent thymocytes achieved by crosslinking the T-cell antigen receptor[19]. Thus it provides a model of the thymocyte death involved in the physiological process of negative selection. Calcium-dependent activation and glucocorticoid sometimes exert mutually antagonistic effects on thymocyte apoptosis[19], suggesting that this *p53*-independent pathway may itself be complex. It may involve protein synthesis, as thymocyte apoptosis induced by either ionophore or glucocorticoid is abrogated by the protein synthesis inhibitor cycloheximide[20]. Cycloheximide also blocks the thymocyte apoptosis caused by radiation[4] and topoisomerase II inhibitors[12], although it does not affect the immediate DNA breakage caused by these agents. The *p53*-dependent and independent pathways may therefore share common elements that require protein synthesis downstream of *p53*. The relationship of the pathways to each other might be resolved through study of p53 substrates (such as the growth-arrest gene *GADD45*; ref. 21) that might be common to both.

In conclusion, we have demonstrated a strictly *p53*-dependent pathway to apoptosis in thymocytes, cells that are oriented

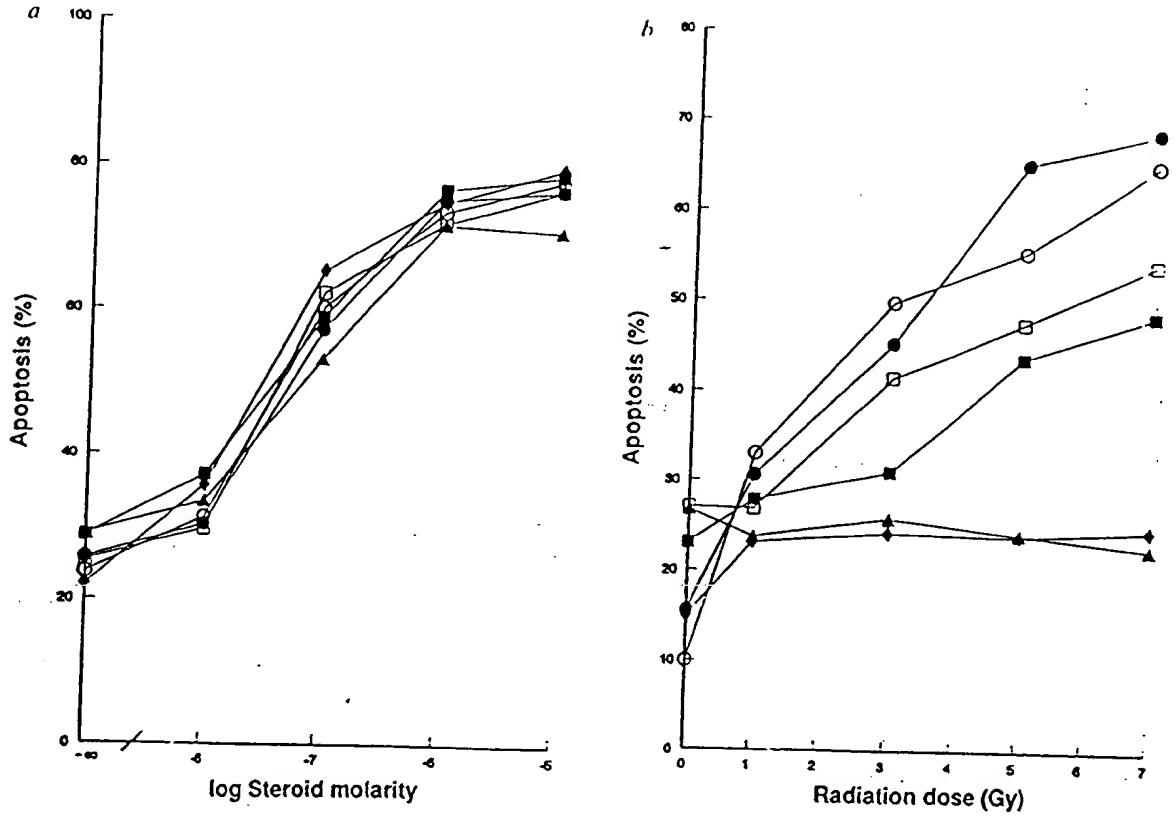

FIG. 2 Induction of apoptosis in thymocytes in suspension culture after 8 h exposure to methylprednisolone (*a*) or 8 h after γ-irradiation (*b*). Cells from the same thymus glands appear in both *a* and *b*, and each thymus is given a unique symbol according to its p53 genotype: wild-type (+/+), filled and open circles; heterozygote (+/−), filled and open squares; homozygous null (−/−), triangles and diamonds.

METHODS. Suspensions of thymocytes (100 μl) at 10^7 ml^{-1} in Glasgow-modified Eagle's medium (GMEM)[3] were placed in the wells of a microtitre plate with methylprednisolone sodium succinate (Solu-medrone, Upjohn) diluted in isotonic saline, or with saline as control. For irradiation, immediately before plating thymocytes were suspended in 1.5-ml screw-capped nylon tubes and exposed to γ-rays from a ^{137}Cs source at ~0.5 Gy min^{-1}. After incubation of the plates at 37 °C in 5% CO_2 in air, cells were fixed in 4% formaldehyde in ethanol overnight, washed three times in water, stained with 10 μg ml^{-1} acridine orange in saline and viewed for nuclear chromatin morphology in a fluorescence microscope. Apoptotic cells were unequivocally recognized by their condensed, sometimes fragmented chromatin[1]. All data points are the means of triplicate wells. At least 200 cells were counted from each well, giving reproducibility to within 5%.

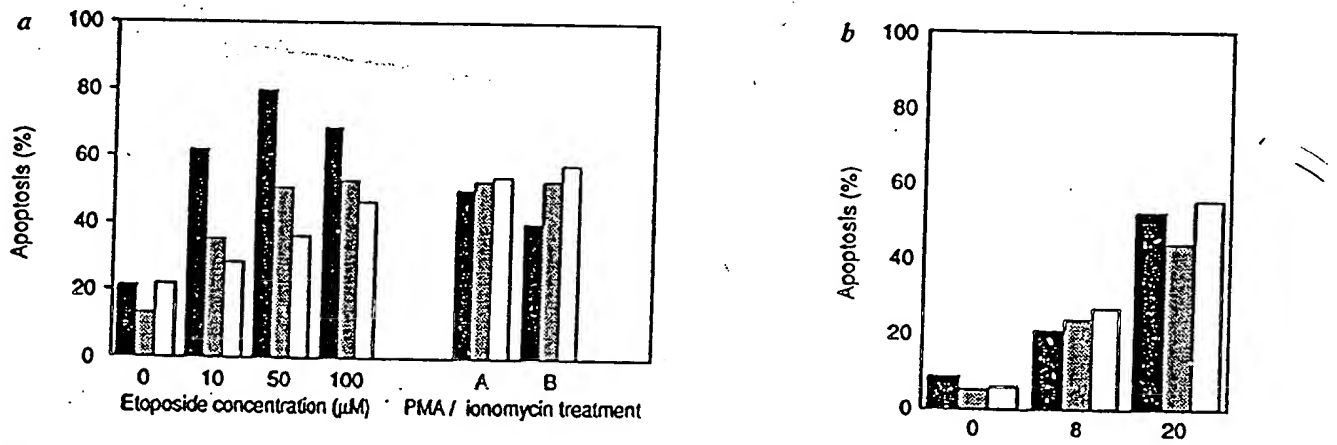

FIG. 3 Induction of apoptosis in thymocytes after 8 h exposure to etoposide or PMA and ionomycin (*a*), or with increasing time in untreated cultures (*b*). The p53 status of these thymocytes was +/+, black bars; +/−, grey bars; −/−, open bars. A single thymus of each type was the source of all the cells in each of *a* and *b*.

METHODS. As for Fig. 2. Etoposide (Sigma) was initially made up in dimethylsulphoxide at 100 mM and diluted in saline. PMA and ionomycin (Sigma) were applied at respective concentrations of 5 ng ml^{-1} and 5 μg ml^{-1} (condition A) or at fivefold greater concentrations of both agents (condition B). The no-treatment control contained 0.1% (v/v) dimethylsulphoxide in saline.

towards programmed deletion by this mode of death. Agents that initiate DNA-strand breakage kill thymocytes by this pathway, but other lethal stimuli are effective in the absence of *p53*. The *p53*-independent stimuli include several that mimic physiological cell-deletion signals, namely glucocorticoid, calcium-associated activation and ageing *in vitro*. □

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Solution structure of the POU-specific DNA-binding domain of Oct-1

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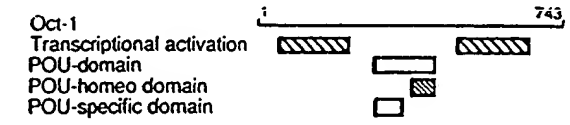
THE transcription factor Oct-1 belongs to a family containing a POU DNA-binding domain. This bipartite domain is composed of a POU-specific domain (POU_s) and a POU-homeodomain (POU_{hd}) connected by a flexible linker. The left half of the optimal POU binding site, the octamer ATGCAAAT, is recognized by POU_s and the right half by POU_{hd}. We have determined the solution structure of POU_s by nuclear magnetic resonance. It consists of four α-helices connected by short loops. Helices I and IV are in a parallel coiled-coil arrangement. The folding topology appears to be similar to that of the bacteriophage λ-repressor and 434 repressor. For the well defined parts of the protein (residues 1–71), the average root-mean square deviation for the backbone atoms is 0.9 Å. Based on the observed selective exchange broadening in the (^{15}N, ^{1}H)-HMQC (heteronuclear multiple quantum coherence) spectrum of the POU_s–DNA complex we conclude that DNA-binding is mediated by helix III. We propose a model for the POU–DNA complex in which both recognition helices from the two subdomains have adjacent positions in the major groove.

POU transcription factors are involved in transcriptional regulation, development and cell differentiation[1–4]. One member of this family, Oct-1, is a ubiquitously expressed protein involved in the regulation of housekeeping genes[5], and adenovirus DNA replication.[6,7] The functional organization of the protein is shown in Fig. 1*a*. The DNA-binding domain consists of two subdomains, which are autonomously folded structures and both display sequence-specific DNA-binding[8,9]. We cloned and expressed an 80 amino-acid fragment containing POU_s, which is functionally active in sequence-specific DNA binding[9]. The amino-acid sequence is depicted in Fig. 1*b*.

The resonance assignment was accomplished using two-dimensional and heteronuclear three-dimensional nuclear magnetic resonance (NMR) spectra of unlabelled and ^{15}N-labelled



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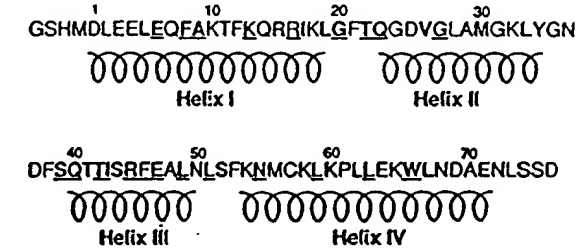

FIG. 1 *a*, Functional organization of the Oct-1 protein[17,18]. *b*, Amino-acid sequence of the POU-specific DNA-binding fragment of Oct-1 used in this work. Residues both underlined and in bold, are absolutely conserved among the POU proteins, whereas residues only in bold, are subject to conservative changes (I/M, I/V/L, R/K, E/D, S/T and S/C; single-letter amino-acid code). The residues 1–71 entail the conserved POU_s domain (corresponding to residues 284 to 355 of Oct-1[18]), whereas residues 72–76 are part of the linker[18]. Residues −4 to −1 arise from the cloning procedure. The secondary structure elements (α-helices) are indicated below the sequence.
METHODS. The protein was expressed by an *Escherichia coli* PET-15[b] plasmid (Novagen). Purification was accomplished by Ni-NTA (Qiagen) affinity chromatography[20]. After thrombin cleavage of the His-tagged protein, POU_s was purified by ion-exchange chromatography to homogeneity. Uniformly ^{15}N-labelled POU_s was produced by using $^{15}NH_4Cl$ as the sole nitrogen source in M9 minimal medium. NMR measurements were done on Bruker AMX 500 and AMXT 600 spectrometers using 2–4 mM solutions of the protein in 0.1 M NaCl, 5 mM dithiothreitol, pH 5.0 at 25 °C.

POU_s (M.C. *et al.*, manuscript submitted). On the basis of the observed sequential and medium range connectivity patterns and the exchange data, four α-helices could be identified (Fig. 1*b*). From the nuclear Overhauser effect (NOE) spectra 53 intraresidual, 372 sequential, 328 medium-range and 263 long-range NOEs were obtained. Furthermore, 82 constraints for 41 hydrogen bonds, and 74 ϕ and 19 χ torsion angle constraints were determined. An overview of all interresidual NOE connectivities is given in Fig. 2.

The structure was determined on the basis of NMR data with the distance geometry program DGII (ref. 10). We used 1,098 upper distance bounds and 93 torsion angle constraints to calculate a set of 30 structures for POU_s, of which 23 had low final energy values after energy minimization and displayed no large violations (see Fig. 3*a*). The structures display an average root-mean square deviation (r.m.s.d.) about the mean coordinate

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Advances in Brief

Increases in Sequence Specific DNA Binding by *p53* following Treatment with Chemotherapeutic and DNA Damaging Agents[1]

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Abstract

We have investigated the effect of chemotherapeutic and DNA damaging agents on binding of the tumor suppressor phosphoprotein *p53* to its consensus DNA sequence. Activation of *p53*-DNA binding was seen for treatment with radiation, hydrogen peroxide, actinomycin D, Adriamycin, etoposide, camptothecin, 5-fluorouracil, mitomycin C, and cisplatin. These results showed that DNA strand breaks were sufficient to lead to increased levels of *p53*. The protein synthesis inhibitor cycloheximide blocks the increase in *p53* following DNA damage. The increase in *p53* activation in camptothecin treated cells may result, at least in part, from an increased half-life of the protein and consequent increases in intracellular protein concentration.

Introduction

The nuclear phosphoprotein *p53* is a tumor suppressor gene involved in cell cycle control (1–4). Mutations of the *p53* locus are among the most common changes seen in human malignancies (5). Loss of wt*p53*,[3] either by deletion or point mutation, increases the susceptibility to tumor formation. This has been observed in patients with inherited defects in the *p53* alleles (Li-Fraumeni syndrome; Ref. 6) and experimentally in transgenic mice with homozygous deletions of the *p53* gene (7). In addition to its established role in the development of malignancy, recent studies indicate that levels of *p53* increase rapidly in response to DNA damage induced by ionizing radiation (1). Cells which incur DNA damage exhibit delays at both the G_1 and G_2 phases of the cell cycle, and these cell cycle delays are thought to allow cells to repair DNA before continuing to DNA synthesis or mitosis. Increased wt*p53* levels are required to initiate the G_1 block, suggesting that wt*p53* is a component of the signal transduction pathway leading to a G_1 arrest (3). In contrast, cells expressing mutant *p53* do not block in G_1 following DNA damage (1). Since mutations in the *p53* gene abolish its ability to bind DNA, it is thought that wt*p53* initiates G_1 arrest through its sequence specific DNA binding.

Among the questions raised by this work, with potential implications for both DNA damage detection mechanisms and therapeutics, are which agents and what types of DNA damage lead to increases in the levels of wt*p53*? The presumed reason for the cell cycle block following DNA damage is to allow time for repair. Consequently, activation of mechanisms for DNA repair may be a component of the DNA damage response and thus will influence how the cell responds to and repairs DNA damage from chemotherapeutic agents. A system involving *p53* may be important or at least serve as a model for cellular responses to DNA damage which might be therapeutically significant. A high percentage of human cancers have a mutant and its presence or absence may influence the efficacy of spec therapies. In addition, determining which particular agents incre wt*p53* may yield information relating to the DNA lesion which tiates the wt*p53* signal transduction pathway.

There is substantial evidence that wt*p53* acts as a regulator transcription and exerts its physiological effect via sequence spec DNA binding (8–11). Consequently, studying the DNA-binding fu tions of wt*p53* may provide important information on the physiol ical function of wt*p53*. A number of specific DNA sequences to wh wt*p53* binds have been identified in genomic DNA (8, 10) and fr *in vitro* studies of binding of wt*p53* to random oligonucleotides (Using these oligonucleotides in conjunction with nuclear extracts s specific *p53* antibodies, we have developed a sensitive EMSA assa detect the presence of endogenous wt*p53*. Using this assay, we sessed how DNA damaging agents altered the levels of wt*p53*-Di binding.

Materials and Methods

Cells. NIH-3T3 mouse fibroblasts were grown in Dulbecco's modif Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% bovine c serum (HyClone, Los Angeles, CA), L-glutamine, and penicillin/streptomy (Gibco, Grand Island, NY). Cells were cultured on 100-mm dishes and u when 80% or more confluent.

Preparation of Nuclear Extract. Following drug exposure or irradiati of the cells, the medium was aspirated and the cells were washed twice w phosphate buffered saline at 4°C. Nuclear extracts were prepared as descrit (9) with the following modifications: cells were lysed by addition of 2.5 ml Buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7 20% glycerol-10 mM NaCl-1.5 mM $MgCl_2$-0.2 mM EDTA-1 mM dithiothreito mM phenylmethylsulfonylfluoride-0.1% Triton X-100), scraped from the d using a rubber scraper, resuspended, and incubated on ice for 5 min. Lysa were centrifuged (800 *g* for 4 min) and the nuclear pellet was resuspended 3 volumes of extraction buffer (Buffer A plus 500 mM NaCl). Nuclei w incubated at 4°C for 30 min with continuous agitation and then centrifuged 35,000 *g* for 10 min. The supernatant containing *p53* was removed for imm diate use or aliquoted and stored at −80°C for subsequent assays.

Electrophoretic Mobility Shift Assays. The consensus *p53* binding s quence determined by Funk (GGACATGCCCGGGCATGTCC; Ref. 9) w synthesized, prepared in double stranded form, and labeled as previous described (12). Binding reactions consisted of nuclear extract (20 μg of pr tein), ^{32}P-labeled oligonucleotide (0.5 ng), salmon sperm DNA (1 μg; Sigm and *p53* antibody (100 ng pAb421; Oncogene Science, Manhasset, NY) wi Buffer A (without the Triton) used to reach a final volume of 25 μl. Bindi reactions were incubated at room temperature for 20 min and 8 μl we analyzed on 4% nondenaturing polyacrylamide gels as previously describ (12).

DNA Damaging Agents. All agents were obtained from Sigma and stor at −20°C after preparation, unless otherwise noted. High concentrations camptothecin precipitated when added to medium. To avoid this, medium w rapidly added to the appropriate quantity of camptothecin solution and th added to the culture dish. All other compounds were added directly to cultu dishes. Stock solutions were prepared as follows: 0.25 mg/ml actinomycin in 95% ethanol; 2.5 mg/ml Adriamycin in phosphate buffered saline; 1 mg/ cisplatin in H_2O, 10 mM camptothecin in DMSO, 10 mM etoposide in DMS

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[3] The abbreviations used are: wt*p53*, wild-type *p53*; EMSA, electrophoretic mobility shift assay(s); DMSO, dimethyl sulfoxide; *gadd*, growth arrest and DNA damage.

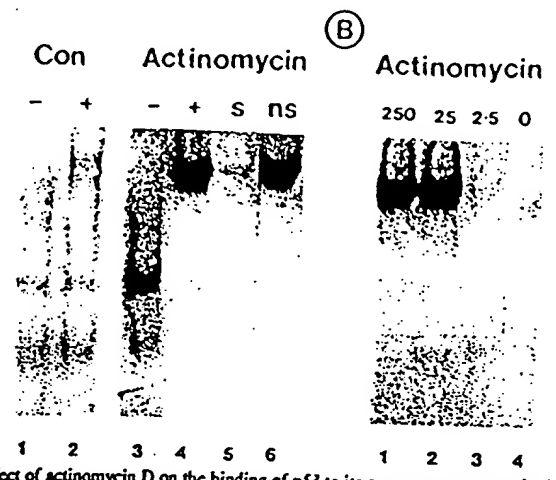

Fig. 1. Effect of actinomycin D on the binding of *p53* to its consensus sequence in the presence and absence of Ab421. In *A*, nuclear extracts from untreated control (*Con*, *Lanes 1* and *2*) or actinomycin treated cells (250 ng/ml for 3 h; *Act*, *Lanes 3–6*) were prepared as described in "Materials and Methods." Extracts were mixed with ^{32}P-*p53* oligonucleotide in the absence (*Lanes 1* and *3*) or presence (*Lanes 2*, *4–6*) of Ab421 (100 ng). For competition assays, binding reactions were supplemented with 100-fold excess of unlabeled *p53* oligonucleotide (*Lane 5*) or a 100-fold excess of a nonspecific oligonucleotide (*Lane 6*). In *B*, cells were exposed to 250 ng/ml (*Lane 1*), 25 ng/ml (*Lane 2*), or 2.5 ng/ml (*Lane 3*) actinomycin D for 3 h or were untreated (*Lane 4*). Nuclear extracts were prepared and EMSA was carried out to detect *p53*-DNA binding as described in "Materials and Methods." *s*, specific; *ns*, nonspecific.

50 mM 5-fluorouracil in H_2O, 1 mM iodoacetamide in H_2O, 1 mg/ml mitomycin C in H_2O, 100 μM taxol in DMSO. Hydrogen peroxide was prepared at a concentration of 100 mM in H_2O on the day of the experiment. Cells were washed in Dulbecco's modified Eagle's medium without serum and incubated in the same prior to addition of the hydrogen peroxide. Radiation was delivered with a ^{137}Cs irradiator at a dose rate of 100 cGy/min. Cycloheximide was prepared as a stock solution of 26 mM in DMSO and stored at –20°C.

Results and Discussion

Fig. 1*A* illustrates EMSA binding of endogenous wt*p53* to its palindromic consensus DNA sequence for both untreated and actinomycin D treated cells. In the absence of antibody 421 (Fig. 1*A*, *Lane 1*) a number of *p53* oligonucleotide-protein complexes were detected in control cells. The addition of anti-*p53* antibody 421 (Ab421) revealed a supershifted band (Fig. 1*A*, *Lane 2*), indicating the presence of wt*p53* in the protein-DNA complexes. In cells treated with actinomycin D, a compound known to increase wt*p53* protein levels (1), Ab421 also revealed a supershifted Ab421-protein complex when compared with extracts without antibody (Fig. 1*A*, *Lanes 3* and *4*). The supershifted band in *Lanes 2* and *4* was seen only with Ab421 and could not be detected with antibodies raised against other portions of the *p53* protein.[4] Additionally, this supershifted band was greatly increased in actinomycin treated cells (compare Fig. 1*A*, *Lanes 2* and *4*). To further characterize the specificity of the interaction of the *p53* consensus sequence with wt*p53*, competition assays were carried out. A 100-fold molar excess of unlabeled *p53* oligonucleotide competitively inhibits *p53* binding (Fig. 1*A*, *Lane 5*, *s*) while an unrelated ("nonself") oligonucleotide had little effect on the *p53*-DNA complex (Fig. 1*A*, *Lane 6*, *ns*). The presence of, and changes associated with, the lower molecular weight nonspecific bands were variable between experiments. Fig. 1*A* demonstrates that specific *p53*-DNA complexes can be detected in nuclear extracts using an antibody specific for wt*p53* and that the levels of this DNA-protein complex can be altered by actinomycin D. This increase was examined further in Fig. 1*B* which indicates that *p53*-DNA binding increases in a dose-dependent manner for actinomycin D concentrations of 2.5 to 250 ng/ml using relatively short (3-h) exposure times.

[4] B. Price, unpublished observations.

In Fig. 2 the effect on *p53*-DNA binding activity of a range of chemotherapeutic and DNA damaging agents was studied. Increases in wt*p53* DNA binding activity was seen for the free radical inducing treatments (radiation, hydrogen peroxide), the combination DNA intercalaters/topoisomerase inhibitors (actinomycin, Adriamycin) and the topoisomerase inhibitors camptothecin and etoposide. The antimetabolite 5-fluorouracil also led to significant but less intense changes as did the alkylating antibiotic agent mitomycin C. Cisplatin treatment gave a small increase, whereas the alkylating agent iodoacetamide did not cause significant increases compared with controls. Concentrations of iodoacetamide one order of magnitude higher did not cause increases in *p53* levels/binding (data not shown). The concentrations used for studies in Fig. 2 were chosen to illustrate the efficacy of each agent, but increased *p53* levels also were observed in response to lower concentrations for most of the compounds, as illustrated for actinomycin D in Fig. 1*B*. For example, significant increases were seen for adriamycin at 0.1 μg/ml and for 5-fluorouracil at 10 μM. Included as a control is the microtubule active agent taxol which does not directly damage DNA but which will cause cell cycle arrest (13). Taxol caused a decrease in *p53*-DNA binding activity compared with untreated cells (Fig. 2). The response to taxol was variable, with other experiments showing slight increases or decreases relative to control (data not shown).

We examined the time course of activation of *p53* for cells treated with two agents operating by different mechanisms: the topoisomerase I inhibitor camptothecin; and the free radical producing agent H_2O_2. The time course of increase in *p53* following treatment with 100 μM camptothecin is shown in Fig. 3*A*. Increases in *p53*-DNA binding were detected as early as 30 min after camptothecin addition and continued for up to 6 h. Exposure of the cells to hydrogen peroxide leads to an increase over 2 h and then a decline to control levels after 6 h. It is unclear why H_2O_2 induction of *p53*-DNA binding should return to control levels after 2–4 h. Cellular metabolism of H_2O_2 may account for some of this decrease. However, transient activation of other transcription factors following peroxide treatment has been observed previously in NIH-3T3 cells (14). Previous studies have shown that increases in wt*p53* protein occur 30 min after irradiation, while for cells exposed to actinomycin there is a slow increase in wt*p53* protein over 24 h (1).

In Fig. 3*B* we have examined the inactivation of wt*p53*-DNA binding activity during recovery from three different stimuli. To do this, cells were exposed to either actinomycin D, camptothecin, or H_2O_2, followed by extensive washing to remove them (Fig. 3*B*). In actinomycin D treated cells, *p53*-DNA binding activity was slightly increased compared to the level at the beginning of the washout (Fig.

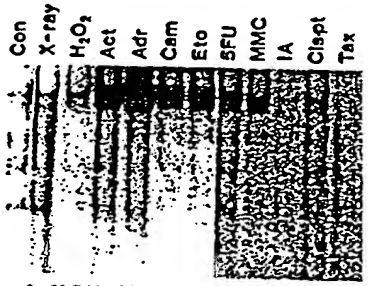

Fig. 2. Stimulation of *p53*-DNA binding by a range of chemotherapeutic/DNA damaging agents. Cells were treated as follows: *Con*, unstimulated controls; *X-ray*, radiation 5 Gy followed by 1 h recovery; H_2O_2, 500 μM for 2 h; actinomycin D (*Act*), 250 ng/ml for 3 h; adriamycin (*Adr*), 10 μg/ml for 3 h; camptothecin (*Cam*), 100 μM for 3 h; etoposide (*Eto*), 400 μM for 3 h; 5-fluorouracil (*5FU*), 50 μM for 3 h; mitomycin C (*MMC*), 10 μg/ml for 24 h; iodoacetamide (*IA*), 10 μM for 3 h; cisplatin (*Cis-pt*), 20 μg/ml for 3 h; taxol (*Tx*), 10 nM for 4 h. Incubations were terminated at the indicated times, nuclear extracts were prepared, and EMSA were carried out as in "Materials and Methods."

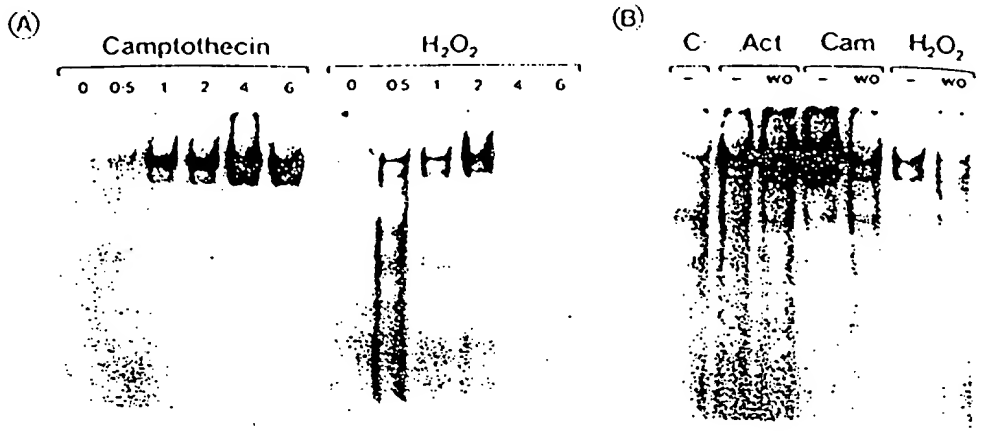

Fig. 3. Time course of activation and reversibility of *p53*-DNA binding activity. *A*, increase in *p53*-DNA binding as a function of time for camptothecin and H_2O_2. Cells were exposed to 100 μM camptothecin or 500 μM H_2O_2 for 0.5, 1, 2, 4, or 6 h or left untreated (*0*) before preparation of nuclear extracts and EMSA. In *B*, cells were incubated for 2 h in actinomycin (*Act*; 250 ng/ml), camptothecin (*Cam*; 100 μM), or H_2O_2 (500 μM). The washout procedure was to rinse cells in serum-free medium followed by fresh serum/Dulbecco's modified Eagle's medium and incubated for 1 h. Washes were repeated 4 times; -, sample prepared at completion of drug exposure; wo, washed.

3*B*). In contrast, when cells treated with camptothecin were washed free of the drug, a significant reduction in the levels of the *p53*-DNA binding activity (Fig. 3*B*) compared to that of unwashed cells (Fig. 3*B*) was seen. In H_2O_2 treated cells, *p53*-DNA binding activity returned to control levels after washout, as expected from previous data. These results are consistent with the known mechanisms of action of the three compounds. Actinomycin is a DNA intercalater whose binding is essentially irreversible, resulting in the chronic *p53* activation observed in these experiments. Camptothecin is a reversible inhibitor of topoisomerase I, the removal of which allows the topoisomerase to complete its catalytic cycle, leading to the observed decreases in *p53* activation. Hydrogen peroxide produces free radicals and single strand breaks in DNA. Once it is removed, DNA breaks may be repaired and the *p53* activity decreased to control levels. In addition, Fig. 3*A* showed that levels decreased at 6 h even if the hydrogen peroxide were left in place.

The above data demonstrate that the increase in *p53* binding activity can be reversed over the course of 4 h if the stimulus is removed. To determine if increased levels of wt*p53*-DNA binding seen here required new protein synthesis or activation of endogenous *p53*, cells were treated with the protein synthesis inhibitor cycloheximide. Fig. 4*A* indicates that exposure of unstimulated cells to cycloheximide eliminates any detectable *p53* signal (*Con* − and +). It also shows that pretreatment of the cells with cycloheximide inhibits completely the increases in *p53* caused by hydrogen peroxide, actinomycin, and camptothecin (Fig. 4*A*; compare *Lanes* − and + for each agent), three agents with different mechanisms for damaging DNA. This result indicates that new protein synthesis, presumably *p53* synthesis, is required in order for cells to increase levels of *p53*. The half-life of *p53*-DNA complex can be determined by adding cycloheximide to prevent new protein synthesis and measuring the levels of *p53* at various times after addition. Treatment of the unstimulated cells gives information on the decay of endogenous *p53*. In Fig. 4*A* (*Con* − and +) *p53*-DNA binding activity was undetectable 2 h after addition of cycloheximide, with an estimate of the half-life of binding of less than 30 min (data not shown). This is consistent with estimates of the half-life of wt*p53* protein previously obtained in unstimulated, untransformed NIH-3T3 cells and found to be about 20 min (15). Thus, the half-life of the binding of the *p53* to DNA in the presence of cycloheximide mirrors the half-life of the protein. The decay of the *p53*-DNA binding activity for camptothecin treated cells in the presence of cycloheximide is shown in Fig. 4*B* and indicates a half-life of hours (compare *Lane* 0 with *Lanes* 2, 4, and 8). There appears to be a relatively rapid decrease over the first 2 to 4 h but not much change between 4 and 8 h after cycloheximide addition. Thus, the nature of the decrease does not allow a precise half-life to be determined. Controls (Fig. 4*B*, *Lane* 5) treated with only camptothecin show higher levels of *p53*-DNA binding at 8 h than do cycloheximide treated cells. For comparison, untreated control cells have a much less intense signal (*e.g.*, compare Fig. 4*A*, *Lanes Con* − and *Cam* −). This

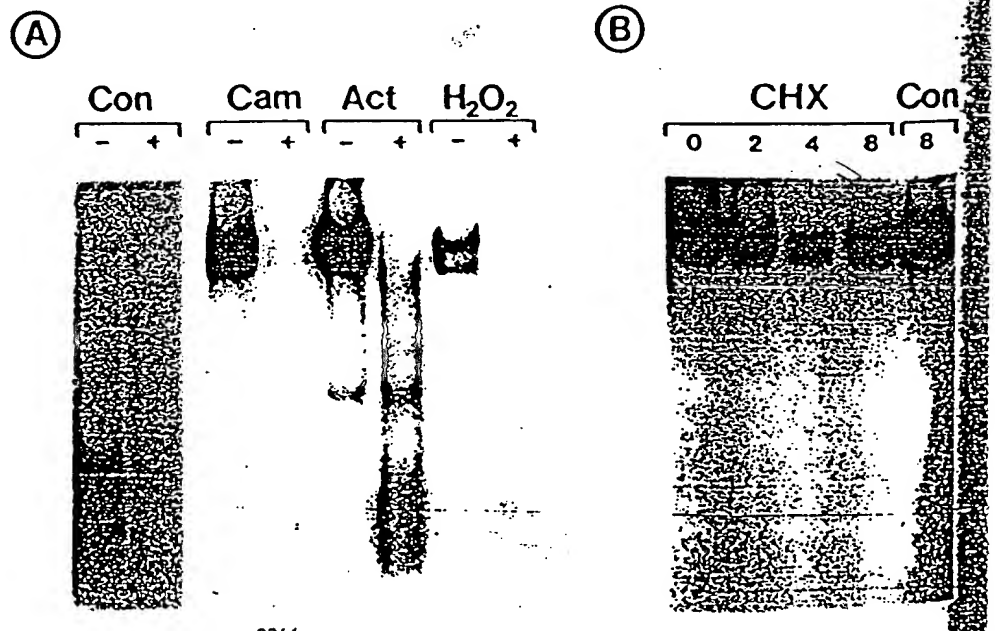

Fig. 4. Effect of cycloheximide on stimulation of *p53*-DNA binding. In *A*, cells were either untreated (−) or pretreated with 13 μM cycloheximide for 15 min (+) prior to addition of the specified compounds. Camptothecin (*Cam*; 100 μM) was added for 3 h, actinomycin D was added for 3 h (*Act*; 250 ng/ml), and H_2O_2 (500 μM) was added for 2 h. Cell extracts were then prepared for EMSA. Control cells (Con) were treated with cycloheximide for 2 h. In *B*, cells were pretreated with 100 μM camptothecin for 2 h prior to the addition of cycloheximide (13 μM). Nuclear extracts were prepared 0, 2, 4, or 8 h later for EMSA. Control (*Con*) cells were exposed to camptothecin but were not treated with cycloheximide.

clearly demonstrates an increase in the half-life of the *p53*-DNA complex (and therefore in the *p53* protein) in camptothecin treated cells.

The data in Fig. 1*A* demonstrate that DNA damaging agents such as actinomycin D induce *p53*-DNA binding, a response which may be secondary to increases in *p53* protein levels shown previously in ML-1 myeloblastic leukemia cells following actinomycin D or radiation treatment (1). The data indicate that EMSA can detect endogenous w*tp53* in nuclear extracts when used in combination with Ab421. Previous studies have noted that Ab421 may influence the interaction of w*tp53* with its DNA recognition site (9, 16). Ab421 interacts with the COOH-terminal region of *p53* (17), which is important in control of the activation of DNA binding. Removal of the 30 COOH-terminal amino acids leads to increased DNA binding and modifications of this portion of the protein may potentially play a role in *in vivo* regulation of *p53* (16). Use of *p53* antibodies recognizing other portions of the protein does not have the same effect on DNA binding and does not function in conjunction with EMSA (3, 9, 16).

Recent studies have examined the relationship between DNA damage and the multiple changes they induce in the cell including *gadd* gene expression, cell cycle arrest, and increased levels of w*tp53* (1–3). One aspect of these interrelated pathways that has not been addressed is what type of DNA damage causes induction of *p53*. Ionizing radiation, actinomycin D (1), UV radiation, and UV-mimetic treatments with 4-nitroquinolone-1-oxide (18) have all been shown to lead to increased levels of w*tp53*. The agents which we showed in Fig. 2 to increase *p53* levels cause DNA damage by a variety of mechanisms. γ-Radiation and peroxide cause DNA strand breaks via free radical formation or direct damage to the DNA (19). Actinomycin D and Adriamycin are DNA intercalaters which inhibit topoisomerase II (20, 21) and additional evidence indicates that actinomycin inhibits topoisomerase I as well (22). Etoposide is an inhibitor of topoisomerase II whereas camptothecin inhibits topoisomerase I (21). All of these agents will lead to strand breaks in DNA due to inhibition of topoisomerase activity. This indicates that single stranded DNA breaks are sufficient to cause increases in w*tp53* levels. Each agent leads to strand break formation by different mechanisms from that for radiation and H_2O_2 but lead to a similar effect on *p53* levels. 5-Fluorouracil acts via multiple pathways including incorporation into RNA, incorporation into DNA, and inhibition of DNA synthesis (20). The absence of a response to the alkylating agent iodoacetamide indicated that this particular type of damage does not induce the signal transduction pathway. This result is in agreement with previous data which showed that alkylating agents do not increase *p53* levels as had UV treatment in a BALB/c 3T3 cell line (18). However, both mitomycin C, which acts as an alkylating agent, and cisplatin, which acts as a DNA cross-linking agent (20), also cause increased levels of *p53*. These data demonstrate that, although single strand breaks are sufficient to cause increased *p53* levels, this is not the only type of DNA damage which leads to increases.

The spectrum of response to DNA damaging agents in terms of *p53* activation differs from other cellular response systems. This is not entirely unexpected since cells react to DNA damage via multiple pathways with distinct initiating events. This is important to note in light of the relationship that has been shown between *p53* activation and expression of the growth arrest gene, *gadd45*. A close correlation has been shown between activation of *p53* and *gadd45* expression (3). By contrast, in a study using HeLa cells with a CAT construct attached to the promoter of a different member of the *gadd* family of proteins, quite different agents induced *gadd153* expression (23). For example, the response to iodoacetamide was comparable to that seen for cisplatin, mitomycin C, Adriamycin, camptothecin, and etoposide (20–30 [times] control) and was roughly twice that seen for peroxide. In contrast, the response to radiation did not differ significantl control. The inactivation of wild-type *p53* in HeLa cells may c ute to this marked difference, or it may be an indication that *ga* is stimulated by a different pathway to that involving *p53*/*gad*

We have also studied the mechanism by which w*tp53* lev increased. Inhibition of protein synthesis completely blocks th vation of *p53* and this suggests that new w*tp53* is synthesi response to DNA damage. The decay of *p53* activity in campto treated cells was greatly increased compared with that of co indicating that stabilization is at least one mechanism for the inc levels following DNA damaging agents. *p53* protein levels a creased in transformed cell lines mainly due to increases in the life of the protein (24). In SV40 transformed cells this was due i to the association of the T-antigen with the *p53* protein (15). vious study showed that for DNA damage induced with UV ir tion, *p53* levels were increased as well as the half-life (from 35 t min; Ref. 18). These results are consistent with the data pres above for cells treated with chemotherapeutic agents.

In conclusion, we have demonstrated increased synthesis and ing of w*tp53* to its consensus DNA sequence after exposure to a of DNA damaging agents. These data indicate that DNA strand b may be sufficient to cause increases in *p53* binding activity. Inc in the activation of w*tp53* appeared to be at least in part rela increases in the cellular concentration of protein. The alkylating iodoacetamide did not lead to increases, but cisplatin and mitor C did, suggesting that other, but not all, types of DNA damage initiate this pathway. The class of DNA damaging agents which their activity by inducing single strand DNA breaks may activ homeostatic mechanism in the cells which results in increased l of w*tp53* bound to its specific DNA sites in the genome. This bi may serve to activate transcription of growth arrest genes *gadd45*; Ref. 3) and/or repress cell cycle genes and halt DNA cation.

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N 522 MOLECULAR CHARACTERIZATION OF A CHORIOCARCINOMA TUMOR SUPPRESSOR GENE, Joseph R. Voland and Christopher Becker, Department of Biology and Cancer Center University of California San Diego La Jolla, California 92093

We have previously reported the cDNA sequence of a transmembrane protein, 721P, expressed on the cell surface of syncytiotropohblast, endothelium, and activated lymphocytes and have shown that it contains a cytoplasmic domain homologous to the *myc* family of oncogenes. This similarity includes a leucine zipper, and basic region approximately 60% homologous to the leucine zipper and basic region of *myc*. (Voland *et al., Proc Nat Acad Sci* 89:10425, 1992).

We have transfected this gene, under the control of a CMV promoter/enhancer, into the choriocarcinoma cell lines BeWo, JEG and JaR, as well as the fibrosarcoma line HT1080. The transfected cells show a reduced proliferative capacity, a decreased mitotic index, and fail to produce tumors in nude mice. Mutational analysis of the cDNA shows that removal of the putative basic region results not only in a loss of the "tumor suppressor" effect, but also in an increase in proliferation of the cells.

We have also utilized an epitope tagging method in which an an epitope of the influenza virus was added to the carboxyl terminus of the cytoplasmic domain of 721P. Preliminary immunohistochemical analysis of transfected cells indicates that the epitope appears to associate with the nuclear membrane, suggesting that at least a portion of 721P could translocate to the nucleus.

These results suggest that the 721P protein may play an important role in the differentiation of several cell types, and that the regions of the molecule homologous to *myc* are important in the function of this molecule. Further mutational analysis and biochemical studies are currently underway to better localize this protein in the cell.

N 523 ASSOCIATION BETWEEN P53 MUTATION AND P-GLYCOPROTEIN EXPRESSION IN BONE AND SOFT TISSUE SARCOMAS, Bun-ichiro Nadayama, Junya Toguchida, Yoshihiko Kotoura and Takao Yamamuro, Department of Orthopaedic Surgery, Kyoto University School of Medicine, Kyoto 606, Japan

The function of the p53 protein in normal cells has not yet been clarified. Recently, however, its activity as a transcriptional regulator was shown in some human genes including the multi-drug-resistance gene, MDR1, encoding P-glycoprotein which plays an important role in the mechanism of drug resistance. In this study, the relationship between p53 gene mutation and expression of P-glycoprotein was analyzed immunohistochemically in various types of bone and soft tissue sarcomas. Of 113 tumors analyzed, 28 (24.8%) showed positive staining for the p53 protein. These included 18 of 67 osteosarcomas, 5 of 20 chondrosarcomas, 4 of 11 malignant fibrous histiocytomas (MFHs) and one Ewing's sarcoma. The expression of P-glycoprotein was also analyzed in 86 of these tumors, and positive staining was found in 27 cases (31.4%); 16 of 55 osteosarcomas, 6 of 18 chondrosarcomas, 3 of 8 MFHs, and one case each of malignant lymphoma and Ewing's sarcoma. Strong correlation was found between the expression of p53 and that of P-glycoprotein; 59.3% of p53-positive tumors expressed P-glycoprotein, whereas P-glycoprotein was detected in 18.6% of p53-negative tumors, the difference being statistically significant (p=0.0004). Close association was much prominent when only the cases with a missense mutation were considered (7 of 9 cases, 77.8%). These data indicate that p53 mutations, especially missense mutations, may closely associate with P-glycoprotein expression *in vivo*, resulting in the tolerance for chemotherapeutic agents.

N 524 TUMOR SUPRESSOR GENE THERAPY OF CANCER: ADENOVIRAL MEDIATED GENE TRANSFER OF p53 AND RETINOBLASTOMA cDNA INTO HUMAN TUMOR CELL LINES. Kenneth N. Wills, Daniel C. Maneval, Patricia Menzel, Suzanto Sutjipto, Shu Fen Wen, Karen Nared-Hood, Matthew P. Harris, Whei Mei Huang, Mei-Ting Vaillancourt, Wendy Hancock, Mia Moulton, Margarita Nodelman and Richard J. Gregory. Canji Inc, 3030 Science Park Rd., San Diego, CA 92121.

Mutations or loss of the p53 and/or retinoblastoma (Rb) tumor suppressor genes are associated with a vast array of human malignancies. Re-introduction of wild-type p53 or Rb into these deficient tumor cells has been shown to suppress their tumorigenic properties, and in some cases, induce apoptosis. Therefore, p53 and Rb gene therapy may be a viable means of treating many types of cancer. To address this, we have constructed a series of adenovirus vectors which direct the expression of either wild-type p53 or Rb. These vectors are deleted for the adenoviral E1a and E1b genes required for viral replication and have substituted in their place expression cassettes in which either tumor suppressor genes are driven by the Ad 2 major late promoter or the CMV promoter. An additional construct in which the Rb gene is driven by its own promoter has also been constructed. Infection of p53 or Rb null/mutant tumor cell lines with these viruses indicate that they can express p53 or Rb, suppress DNA replication, affect cell growth and induce apoptosis in some cases. Our results suggest that adenovirus mediated tumor suppressor gene transfer may be an effective treatment for certain types of cancer. We are currently testing this hypothesis in animal models.

N 525 HETEROGENEITY OF RB1 GENE EXPRESSION IN OSTEOSARCOMA, Jiaxiang Zhao[1], Hong-Ji Xu[2], Shi-Xue Hu[2], A. Kevin Raymond[3], Robert S. Benjamin[4], William F. Benedict[2], and Marc F. Hansen[1], Departments of [1]Molecular Genetics, [3]Pathology, and [4]Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030 and [2]The Center for Biotechnology, Baylor College of Medicine, The Woodlands, TX.

Osteosarcoma is the most common primary malignancy of bone in children and adolescents. The current classification of osteosarcoma is based on three general criteria: differentiation of the tumor cells, type of matrix produced by the tumor cells, and anatomical location of the tumor. Osteosarcoma can be broadly divided into two histological categories based on incidence: conventional osteosarcoma and variant histology osteosarcoma. Loss of heterozygosity and functional inactivation of the retinoblastoma susceptibility (RB1) gene is a common feature in conventional histology osteosarcoma occurring in 75% or more of tumors. Well differentiated intraosseus osteosarcoma is one of the variant histology osteosarcomas with a distinct clinical behavior from conventional histology osteosarcoma. Analysis of well differentiated intraosseus osteosarcomas revealed no loss of heterozygosity at the RB1 gene. Further analysis by in situ immunohistochemistry revealed normal RB1 protein expression suggesting that these tumors arise by a different genetic mechanism than conventional histology osteosarcoma.

DZ 304 IMMUNIZATION WITH CYTOKINE-SECRETING ALLOGENEIC MOUSE FIBROBLASTS EXPRESSING MELANOMA ASSOCIATED ANTIGENS PROLONGS THE SURVIVAL OF MICE WITH MELANOMA, Edward P. Cohen and Tae Sung Kim. Department of Microbiology, University of Illinois at Chicago, Chicago, IL 60612

Immunization of C57BL/6 mice (H-2^b) with a mouse fibroblast cell-line of C3H origin (H-2^k) genetically modified for IL-2-secretion and the expression of melanoma associated antigens (MAA) resulted in a specific, generalized anti melanoma response that was capable of prolonging the survival of mice with established melanoma. Both Lyt-2.2$^+$ (CD8$^+$) cells and NK/LAK cells with anti melanoma cytotoxicity were responsible. The anti melanoma immunity was insufficient to fully eradicate all the neoplastic cells, however, and tumor growth eventually recurred. Cells from the recurrent neoplasms formed melanin were histologically indistinguishable from melanoma cells in C57BL/6 mice injected with B16 cells alone. Cells from the recurrent melanomas were resistant to additional rounds of immunotherapy, as indicated both by cytotoxicity assays performed *in vitro* and by the lesser periods of survival of mice with established recurrent melanomas treated with the IL-2-secreting cell construct. The resistant melanoma cells were MHC Class I-deficient. The potential for combination immunotherapy was indicated by the survival of mice with melanoma immunized with allogeneic fibroblasts modified to secrete both IL-2 and interferon-gamma. It was significantly longer than that of mice immunized with cells modified for secretion of either cytokine alone.

DZ 305 GENE THERAPY FOR PHENYLKETONURIA (PKU): PHENOTYPIC CORRECTION IN A MOUSE MODEL BY ADENOVIRUS-MEDIATED HEPATIC GENE TRANSFER . ((B. Fang[1], R. C. Eisensmith[2], X. H. C. Li[4], A. Shedlovsky[3], W. Dove[3] and S. C. L. Woo[1,2,4].)) [1]Howard Hughes Medical Institute, [2]Department of Cell Biology and [4]Institute of Molecular Genetics, Baylor College of Medicine, Houston, Texas and [3]Department of Oncology, University of Wisconsin Medical School, Madison, Wisconsin.

Classical phenylketonuria (PKU) predisposes affected individuals to severe mental retardation and is caused by a deficiency of hepatic phenylalanine hydroxylase (PAH). The initiation of a low-phenylalanine (PHE) diet early in life can reduce or prevent the mental retardation characteristic of this disease. However, the success of this therapy can be limited by poor compliance, especially in older individuals. We have therefore examined the potential application of somatic gene therapy for the phenotypic correction of PKU. A recombinant adenoviral vector containing the human PAH cDNA (Adv/RSV-hPAH) was constructed and administered to PAH-deficient mice (strain Pah^{enu2}). These animals exhibit a biochemical phenotype similar to human PKU patients, with extremely high serum PHE levels and hepatic PAH activities less than 1% of that in wild-type animals. Both hepatic PAH activity and serum PHE levels in these animals were completely normalized five days after treatment, but remained unchanged following infusions of a similar number of particles of a control adenovirus. Although this therapeutic effect did not persist, these studies conclusively demonstrated that only 10%-20% of normal enzymatic activity in the mouse liver is sufficient to restore normal plasma phenylalanine levles. These results suggest that PKU and other metabolic disorders secondary to hepatic deficiencies can be corrected in the future by gene therapy with long-lasting vectors.

DZ 306 DEVELOPMENT OF A COMPETITIVE RT-PCR ASSAY FOR THE QUANTITATIVE DETECTION OF gp91 GENE EXPRESSION IN PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE

Mauro Giacca[1], Lorena Zentilin[1,2], Gabdele Grassi[1], Sabina Taturo[1], Alessandro Ventura[2], and Arturo Falaschi[1]; [1]International Centre for Genetic Engineering and Biotechnology,Trieste (Italy); [2]Children Hospital *Burlo Garofolo*, Trieste (Italy)

We are interested in the development of a gene therapy protocol for the X-linked form of chronic granulomatous disease (CGD), an inherited disorder resulting from the inability of phagocytic cells to produce superoxide anion (O_2^-) due to the absence or to the non functionality of the gp91 protein, a component of cytochrome b245.

We have studied the genetic defect in four Italian patients with the disease, and established lymphoblastoid cell lines by EBV infection, which were used for Southern and Northern hybridization analysis. No gross abnormalities could be detected in the DNA of the four patients; the amount of gp91 mRNA was present in normal levels in three patients, and was undetectable in the fourth one.

We have developed a competitive RT-PCR assay to precisely quantify the amount of gp91 mRNA. This assay is based on the addition to the RNA sample of a competitor RNA fragment having exactly the same sequence as the target transcript, except for the addition of 20 nt in the middle. By this method, all variables which affect reverse transcription and amplification of the target RNA have the same effect also on the competitor. The absolute amount of RNA molecules in the sample is determined by the ratio between the two amplification products. The competitor RNA was obtained by a recombinant PCR technique followed by in vitro transcription [Diviacco et al., 1992, Gene 122, 313-320] [Menzo et al., 1992, J. Clin. Microbiol. 30, 1752-1757].

This strategy has allowed:

- to detect the presence of low levels of transcription (about 100 times less than the normal ones) even in the patient which scored negative in Northern blotting;
- to directly measure the amount of gp91 mRNA in the peripheral blood granulocytes of the patients. This approach indicated that the physiological level of transcription is about 10^4 times less than the one of the ß-actin mRNA; the amounts of transcripts detected in the granulocytes precisely reflect the ones found in immortalized B-cell lines;
- to screen for the efficiency of gp91 mRNA expression in the packaging cell line used for the production of an amphotropic retrovirus; this retrovirus was constructed by the insertion of the coding region of the mRNA in the pBabe-lyg vector;
- to monitor the expression of the gp91 mRNA in retrovirus-infected B-lymphoblastoid cell lines from the patients.

DZ 307 TUMOR SUPPRESSOR GENE THERAPY OF CANCER: ADENOVIRAL MEDIATED GENE TRANSFER OF THE p53 cDNA INTO HUMAN TUMOR CELL LINES, Richard J. Gregory, Daniel C. Maneval, Suganto Sutjipto, Shu Fen Wen, Matthew P. Harris, Karen Nared-Hood, Patricia Menzel, Whei Mei Huang, Mei-Ting Vaillancourt, Wendy Hancock, Mia Moulton, Margarita Nodelman and Kenneth N. Wills, Canji Inc, 3030 Science Park Rd, San Diego, CA, 92121.

Mutation or loss of the p53 tumor suppressor gene is the most frequently detected genetic alteration associated with human malignancies. Introduction of wild-type p53 into p53 altered human tumor cells suppresses their tumorigenic properties and in some cases induces apoptosis. In principle p53 gene therapy would seem to be a viable means of treating many cancers, and we have therefore constructed a series of adenovirus vectors which direct expression of the human wild-type p53. These vectors are deleted for the adenoviral E1a and E1b genes required for viral replication and have substituted in their place expression cassettes in which p53 expression is driven from the adenovirus major late promoter or the CMV promoter. Infection of p53^{null} or p53^{mut} tumor cell lines with these viruses or appropriate controls indicates that they can express p53, suppress DNA replication, inhibit cell growth and induce apoptosis in certain cell lines. Other human tumor cells appear to be refractory to these effects. Interestingly, preliminary evidence suggests that those cells which do not respond to the p53 adenoviruses may not be efficiently infected by Ad5 based vectors. These results suggest that adenovirus mediated p53 gene transfer may be an effective treatment for certain cancers, a hypothesis we are currently testing in animal models.

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Participation of p53 Protein in the Cellular Response to DNA Damage[1]

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ABSTRACT

The inhibition of replicative DNA synthesis that follows DNA damage may be critical for avoiding genetic lesions that could contribute to cellular transformation. Exposure of ML-1 myeloblastic leukemia cells to nonlethal doses of the DNA damaging agents, γ-irradiation or actinomycin D, causes a transient inhibition of replicative DNA synthesis via both G_1 and G_2 arrests. Levels of p53 protein in ML-1 cells and in proliferating normal bone marrow myeloid progenitor cells increase and decrease in temporal association with the G_1 arrest. In contrast, the S-phase arrest of ML-1 cells caused by exposure to the anti-metabolite, cytosine arabinoside, which does not directly damage DNA, is not associated with a significant change in p53 protein levels. Caffeine treatment blocks both the G_1 arrest and the induction of p53 protein after γ-irradiation, thus suggesting that blocking the induction of p53 protein may contribute to the previously observed effects of caffeine on cell cycle changes after DNA damage. Unlike ML-1 cells and normal bone marrow myeloid progenitor cells, hematopoietic cells that either lack *p53* gene expression or overexpress a mutant form of the *p53* gene do not exhibit a G_1 arrest after γ-irradiation; however, the G_2 arrest is unaffected by the status of the *p53* gene. These results suggest a role for the wild-type p53 protein in the inhibition of DNA synthesis that follows DNA damage and thus suggest a new mechanism for how the loss of wild-type *p53* might contribute to tumorigenesis.

INTRODUCTION

Exposure to DNA damaging agents probably contributes to the development of many human cancers (1). Therefore, much effort has been focused on understanding how cells respond to DNA damage and restore the linear DNA sequence integrity and chromatin structure. An important component of the cellular response to DNA damage is an inhibition of replicative DNA synthesis (*e.g.*, Refs. 2–6). Presumably, this response allows optimal repair of damage before the cell reinitiates replicative DNA synthesis and/or begins mitosis. If the damage were not repaired before initiation of S-phase, the use of a damaged DNA template during replicative synthesis could "fix" and propagate mutagenic lesions that might contribute to cellular transformation (7). The inhibition of replicative DNA synthesis after DNA damage may be a critical step in avoiding the progressive increase in genomic changes that characterizes neoplastic transformation (8, 9). Furthermore, cells that are inefficient at this inhibitory process may be prone to neoplastic development. We therefore sought to characterize some of the mechanisms in mammalian cells that control the cell cycle changes in response to DNA damage.

The inhibition of DNA synthesis that follows DNA damage could be achieved through inhibition of a positive (stimulatory) regulator pathway of DNA synthesis and/or stimulation of a negative regulator pathway. Since recent data suggest that the wild-type *p53* gene product plays a role in the inhibition of DNA synthesis (10–13), and since this "growth/tumor suppressor" gene is the most commonly mutated gene thus far identified in human cancers (14), with abnormalities of the *p53* gene present in a wide spectrum of cancers, including tumors of the breast, lung, colon, bladder, brain, bone, hematopoietic, and muscle tissues (reviewed in Ref. 15), we examined the possibility that *p53* might be a negative regulator in this cellular response to DNA damage.

Unfortunately, because of its extremely short half-life (16–19) and its growth inhibitory effects (10–12, 20, 21), studies of the roles and mechanisms of action of the normal, wild-type p53 protein have been extremely difficult. We have recently developed a sensitive and specific flow cytometric assay for quantitating relative levels of endogenous p53 protein in human hematopoietic cells (22). We utilized this assay to assess changes in the levels of p53 protein after DNA damage. We also evaluated changes in cell cycle progression after DNA damage in cells with normal or abnormal expression of *p53*.

We observed increases in p53 protein levels in proliferating normal human bone marrow progenitor cells and in ML-1 myeloblastic leukemia cells after DNA damage, apparently occurring via a posttranscriptional mechanism. A similar rise in p53 protein levels had previously been noted in mouse 3T3 fibroblasts after UV irradiation (23). With 2 different DNA damaging agents, this rise in p53 protein levels in ML-1 cells was temporally correlated with a transient G_1 arrest. Both the increase in p53 protein levels and the decrease in DNA synthesis (specifically, the G_1 arrest) after DNA damage were blocked by the protein synthesis inhibitor, cycloheximide, and by the phosphodiesterase inhibitor, caffeine. In contrast to cells with no detectable *p53* gene mutations (ML-1 and normal human bone marrow myeloid progenitors), cells with mutant *p53* genes (Raji and-RPMI 8402 lymphoid leukemia cells and KG-1a myeloid leukemia cells) or with no *p53* genes (HL60 myeloid leukemia cells) continued to progress through S-phase after DNA damage. These results suggest that wild-type p53 protein may play a role in the inhibition of DNA synthesis that follows DNA damage.

MATERIALS AND METHODS

Cells and DNA Damage. Cell lines were grown in RPMI 1640 containing 10% serum. Human hematopoietic progenitor cells (CD34+) from normal volunteers were isolated by immunomagnetic adherence as described previously (24). Myeloid progenitor cells from this isolated cell population were grown in a liquid culture system containing HL-1 medium (Ventrex Laboratories, Portland, ME) with 30% fetal calf serum and 7% supernatant from the bladder carcinoma cell line 5637 for maximal growth. After 4 days in culture, these normal bone marrow progenitor cells reached logarithmic phase growth, and cell cycle changes after γ-irradiation were assessed. Cultured cell lines or myeloid bone marrow cells were exposed to a selected dose of γ-irradiation, actinomycin D, or ara-C[3] (continuous exposure over the time course for the chemical agents) and evaluated at various times

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[3] The abbreviations used are: ara-C, 1-β-d-arabinofuranosylcytosine; IFA, 10 mM 4-(2-hydroxyethyl)-1-peperazinethane sulfonic acid, pH 7.4, 150 mM NaCl, 4% newborn calf serum, 0.1% NaN_3; MFI, mean fluorescence intensity; BrdUrd, bromodeoxyuridine; XRT, γ-irradiation; CHX, cycloheximide.

afterwards for changes in *p53* levels or cell cycle progression. Cell viability was greater than 90% by trypan blue exclusion up to 96 h after DNA damage for virtually all doses and agents used. Cells were irradiated in their flasks in media in a ^{137}Cs γ-irradiator for an appropriate length of time to deliver a preselected dose (usually at ~100 rads/min). When desired, caffeine was added 30 min before irradiation. In each case, control cells were handled in the same way with only the omission of the DNA damaging agent.

Flow Cytometric Assay for *p53* Expression. Hybridoma cells secreting the anti-*p53* antibody, p421, were generously provided by Dr. Arnold Levine. A specific inhibitory peptide (25) was synthesized and purified by The Johns Hopkins University School of Medicine Protein/Peptide Facility. Flow cytometric analysis of p53 protein was carried out as described previously (22). Briefly, cells were fixed by dropwise addition of 70% methanol and incubation for 5 min at -20°C, washed, and then incubated with 0.1 ml p421 hybridoma supernatant [diluted 1:5 in IFA buffer with or without the presence of 0.5 μg of the purified specific inhibitory peptide (previously titrated for maximal inhibition). After another wash, the cells were incubated with goat anti-mouse IgG2a fluorescein isothiocyanate (diluted 1:500 in IFA; Southern Biotechnology Associates, Birmingham, AL) for 30 min, washed in IFA twice, and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Nonspecific blocking serum (2% goat and human) was present during each antibody incubation. All procedures after fixation were done at 4°C. Raji and HL-60 cells were run in every experiment as positive and negative controls, respectively, for p53 protein expression. Peptide-inhibitable fluorescence is present only in cells expressing p53 protein, and the amount of fluorescence correlates well with the amounts of p53 protein assessed by immunoblot or immunoprecipitation (22). Relative levels of p53 protein were evaluated by determining a "corrected *p53* MFI" as described previously (26). [Corrected MFI equals the difference between the MFI of antibody binding and antibody plus inhibitory peptide; using the p421 antibody, it has previously been shown to accurately reflect the presence of p53 protein (22).]

Northern Blots and Immunoprecipitations. Total cellular RNA was isolated using the guanidine isothiocyanate/cesium chloride procedure (27). Electrophoretic separation, RNA transfer to nylon membranes, hybridization, and autoradiographic identification were done by standard techniques (28). Filters were hybridized successively to *p53* and β-actin probes labeled with [^{32}P]dCTP by the random primer method (29). For immunoprecipitations, cells were incubated in methionine-free RPMI 1640 with 20% dialyzed fetal calf serum for 1 h before labeling cells with 100 μCi/ml [^{35}S]methionine for 2 h. After washing, nuclei were isolated by hypotonic lysis according to the method of Klempnauer and Sippel (30), and *p53* was extracted from the nuclear pellets in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 0.005% leupeptin, 200 units/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, and immunoprecipitated with either p421 antibody or control antibody, p2037 (a nonspecific IgG2a) bound to protein A-Agarose (Sigma) after preclearing the lysate × 2 with p2037, protein A-Agarose, and sansorbin (Calbiochem, La Jolla, CA). Immunoprecipitated proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was dried and autoradiographed using preflashed film after fluorographic amplification (Amersham).

Cell Cycle/Proliferation Assays. DNA synthesis was assessed by incorporation of BrdUrd and flow cytometric analysis using the method described by Hoy *et al.* (31). Briefly, after incubation with 10 μM BrdUrd for 4 h, cells were fixed in 70% methanol as above, resuspended in 0.1 N HCl/0.7% Triton X-100 for 10 min at 4°C, washed with excess phosphate-buffered saline, and heated to 97°C for 10 min in deionized acidified water. The cells were then chilled in ice water for 10 min and washed twice with IFA buffer containing 0.5% Tween 20. After incubation with 0.1 ml anti-BrdUrd-fluorescein isothiocyanate (1:5 in IFA buffer, 30 min, 4°C; Becton Dickinson), the cells were washed twice and then analyzed by flow cytometry. For simultaneous analysis of DNA synthesis and cell cycle, after BrdUrd staining and washing, the cells were treated with 50 units of RNase (Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min and incubated for at least 1 h with 25 μg/ml propidium iodide (Sigma) in phosphate-buffered saline, pH 7.4.

p53 Gene Sequencing. DNA was isolated from cell lines, and exons 5 through 9 of the *p53* gene were amplified from these DNA samples through the use of the polymerase chain reaction. These products were then subcloned into Lambda Zap (Stratagene) and double-stranded plasmid was obtained. Pooled plasmids were then sequenced and compared with the normal sequence of the *p53* gene as described previously (32).

RESULTS

Alterations in Cell Cycle Progression and *p53* Expression in ML-1 Cells after γ-Irradiation. We have previously demonstrated that the changes in p53 protein expression during proliferation and differentiation of ML-1 myeloid leukemia cells closely parallel those in normal human bone marrow progenitor cells (22). In addition, sequencing of exons 5 through 9 of the ML-1 *p53* gene revealed no mutations (see below). Thus, ML-1 cells appear to be a useful model for investigations of the physiological functions of endogenous, wild-type p53 protein.

Exposure of ML-1 cells to nonlethal doses of XRT results in a dose-dependent, transient decrease in replicative DNA synthesis (Fig. 1). Changes in the cell cycle progression of these cells after DNA damage were assessed by simultaneous flow cytometric analysis of DNA synthesis (by BrdUrd incorporation) and of DNA content (by propidium iodide staining). This analysis can detect a decrease in the number of cells entering S-phase as early as 3 h after XRT (Fig. 1*B*; *see arrows*), even though the change in the percentage of S-phase cells in the total cell population is small at this time (Fig. 1*A*). The decrease in the number of S-phase cells is due to cells arresting in both G_1 and G_2/M (Fig. 1, *B* and *C*). G_1 arrest predominates at low doses of XRT (<100 rad), whereas the G_2 arrest becomes more prominent at higher doses (data not shown). Cells that are already in S-phase at the time of DNA damage appear to continue to progress through to G_2/M, whereas cells in G_1 do not continue to enter S-phase (Fig. 1*B*).

Expression of p53 protein in ML-1 cells after XRT was evaluated by both flow cytometry (Fig. 2, *A* and *C*) and metabolic labeling/immunoprecipitation (Fig. 2*B*). Increases in p53 protein levels were seen within 1 to 2 h after XRT with both techniques. A similar increase in p53 protein levels was seen in proliferating normal bone marrow progenitor cells after XRT (data not shown). We have previously demonstrated the specificity of the p421 antibody and peptide inhibition in the flow cytometric assay for detecting p53 protein (22). The specificity of these assessments of changes in p53 protein levels after XRT is demonstrated here by specific peptide inhibition in the flow cytometric assay [Fig. 2*A*; antibody plus peptide gives identical levels of fluorescence as a control antibody (data not shown)] and the use of a control IgG2a antibody in the immunoprecipitation assay (Fig. 2*B*). In addition, the increase in anti-*p53* antibody binding to ML-1 and bone marrow progenitor cells in the flow cytometric assay could not be attributed to some nonspecific binding simply attributable to DNA damage since no increases in antibody binding were observed in cells with no *p53* expression (HL-60 and KG-1a) or expression of mutant p53 protein (Raji) (Fig. 2*C* and see below).

The increases in p53 protein levels were detectable within 30 min after XRT and returned towards normal levels about 48 to 72 h later (Fig. 3). This time course of increases in p53 protein levels correlated well with the cell cycle changes, which became detectable within 3 h and returned towards normal 48 to 72 h

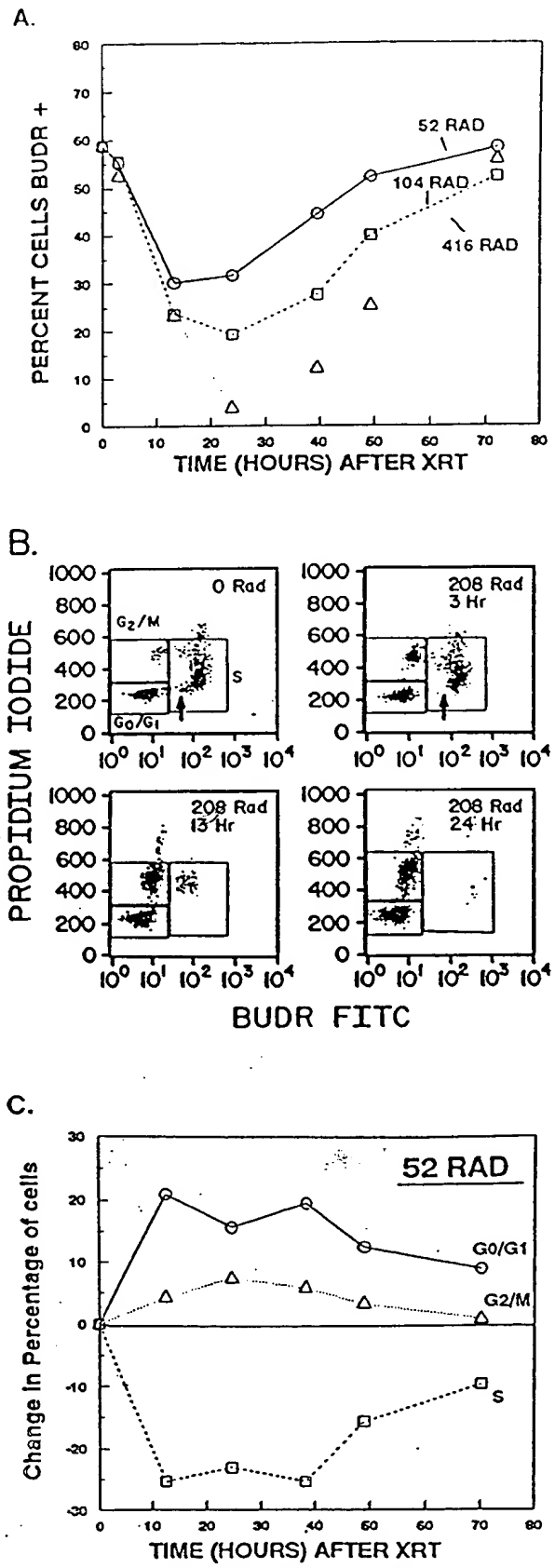


Fig. 1. Time course of cell cycle changes in ML-1 cells exposed to XRT. *A*, DNA synthesis after various doses of XRT was assessed by flow cytometric analysis of BrdUrd (BUDR) incorporation during a 4-h pulse at various times after exposure to 52, 104, or 416 rads. *B*, cell cycle distributions in control cells (0 rad) or cells at 3, 13, and 24 h after exposure to 208 rads. Flow cytometric dot plots display simultaneous analysis of S-phase DNA synthesis (determined after a 4-h pulse with BrdUrd at the various times after XRT) on the *ordinate* and DNA content (determined by staining with propidium iodide) on the *abscissa*. Cell cycle populations are characterized as G_0/G_1 (2N DNA content with no BrdUrd incorporation), S-phase (variable DNA content with BrdUrd incorporation), and G_2/M (4N DNA content with no BrdUrd incorporation during the pulse period). *Arrows*, area containing cells that have recently entered S-phase from G_1. *C*, quantitation of the changes in the percentage of cells in each cell cycle phase (assessed as in *B*) at various times after exposure to 52 rads.

later (Fig. 1). Increases in p53 protein were detectable with doses as low as 52 rad (data not shown) and, in most experiments, 3- to 5-fold increases in the mean fluorescence intensity of p53 protein were noted at doses between 52 and 208 rad. Similarly, densitometer tracings of immunoprecipitated [^{35}S] methionine-labeled p53 protein revealed 3- to 5-fold increases within 1 h after XRT (data not shown). Furthermore, similar to the dose dependence noted for the degree of inhibition of DNA synthesis after XRT (Fig. 1*A*), levels of p53 protein also increased more at higher doses, at least between 52 and 416 rad (data not shown).

To begin to elucidate the mechanism of the induction of p53 protein after DNA damage, total cellular RNA was isolated from ML-1 cells at various times after various doses of γ-irradiation. Northern blot analyses revealed no significant changes in the levels of *p53* mRNA (Fig. 4). This observation suggests that the changes in p53 protein after DNA damage result from post-transcriptional mechanisms and is consistent with the previous report of an increased half-life of p53 protein in 3T3 cells after UV damage (23).

Effects of Other Cytotoxic Agents on Cell Cycle and *p53* Expression in ML-1 Cells. Actinomycin D is a DNA intercalating agent that, like γ-irradiation, induces DNA strand breaks (33, 34). In contrast, ara-C is an S-phase-specific anti-metabolite that does not directly damage DNA (35). Treatment of ML-1 cells with actinomycin D causes a G_1 arrest (36) and induces a significant increase in p50 protein levels (Fig. 5*A*), though the time at which the increase is noted is much later than that seen after γ-irradiation. However, the decrease in replicative DNA synthesis after actinomycin D treatment also occurs later than after XRT (compare Figs. 5*B* and 1*A*), and thus still temporally coincides with the increase in p53 protein. In contrast, treatment with ara-C induces an S-phase arrest [though it does not decrease the number of cells that enter S-phase (Fig. 5*B*)], and no significant changes in p53 protein levels are observed (Fig. 5*A*). Thus, it appears that p53 protein levels increase after exposure to agents that damage DNA and cause a G_1 arrest, such as XRT and actinomycin D.

Effects of Caffeine and Cycloheximide on Cell Cycle Changes and *p53* Expression after DNA Damage. Caffeine treatment potentiates the cytotoxicity of DNA damaging agents by preventing the inhibition of DNA synthesis (2, 37, 38). We found that while exposure of ML-1 cells to caffeine caused an increase in the percentage of undamaged cells in G_1 (Fig. 6*A*, *left column*), caffeine treatment inhibited the usual decrease in DNA synthesis in cells exposed to XRT [Figs. 6*A* (*right column*) and 7*A*]. We reasoned that if p53 protein actively participates in this inhibition of replicative DNA synthesis after DNA damage, then the increases in p53 protein usually seen after DNA damage might also be affected by caffeine treatment. Exposure of cells to 4 mM caffeine, which inhibited the decrease in DNA synthesis after DNA damage (Figs. 6*A* and 7*A*), also blocked the increase in p53 protein levels (Figs. 6*B* and 7*B*). These data suggest that some of the previously described effects of caffeine on cells after DNA damage may be due to a block of p53 protein induction and implicate alterations in cyclic

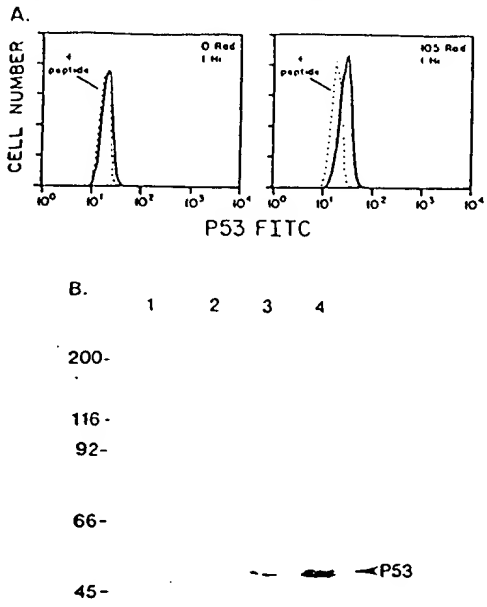


Fig. 2. Levels of p53 protein in ML-1 cells exposed to XRT. *A*, flow cytometric analysis of p53 protein expression 1 h after exposure to 0 or 105 rads. —, Fluorescence histograms after staining with anti-*p53* antibody, p421; · · · ·, control (antibody preincubated with inhibitory peptide) for the same condition. A shift to the right of the histograms in the p421-stained samples indicates the presence of p53 protein. *B*, immunoprecipitation of [^{35}S]methionine-labeled p53 protein 2 h after exposure of ML-1 cells to 0 rad (*Lanes 1* and *3*) or 206 rad (*Lanes 2* and *4*). Nuclear proteins were immunoprecipitated with control antibody, p2037 (*Lanes 1* and *2*), or p421 (*Lanes 3* and *4*). Extracts from equivalent numbers of cells (75 × 10^6 cells/lane) and equivalent numbers of ^{35}S counts (after preclearing of the extracts) were immunoprecipitated for each sample. Molecular weight standards (K_d) are shown on the *left*. *C*, flow cytometric analysis of p53 protein expression (binding of p421 antibody) 2 h after exposure of ML-1, HL-60, KG-1a, or Raji cells to 0 rad (—) or 208 rad (· · · ·). FITC, fluorescein isothiocyanate.

nucleotide levels in this regulatory process.

Brief exposures of ML-1 cells to the protein synthesis inhibitor, CHX, similarly blocked the induction of p53 protein (within 2 h; Fig. 8*B*) and partially inhibited the G_1 arrest measured 15 h after XRT (Fig. 8*A*). [The rapid reversibility of the effect of a pulse of CHX on p53 protein levels (see below) is consistent with the partial inhibition of the G_1 arrest observed in this experiment]. These results demonstrate that the G_1 arrest after DNA damage is an active cellular response dependent on new protein synthesis and/or on a short-lived protein. (The rapid disappearance of all detectable p53 protein after CHX treatment is also consistent with the short half-life of wild-type p53 protein.) The inhibition of p53 protein induction by CHX was reversible, however, since p53 protein levels increased rapidly and the the G_1 arrest was re-initiated after a washout of the CHX from XRT-treated cells (data not shown). Interestingly, as previously noted in yeast (39), the G_2 arrest was not significantly affected by CHX treatment (Fig. 8*A*). These observations with caffeine and cycloheximide further link the changes in p53 protein levels to the inhibition of replicative DNA synthesis after DNA damage. In addition, they indicate that the G_1 arrest is mediated by a physiological control mechanism, rather than by structural constraints of damaged DNA, in agreement with suggestions by Weinert and Hartwell (6).

Cell Cycle Changes after DNA Damage in Cells with Abnormal *p53* Genes. The data presented thus far demonstrate a strong correlation between decreased DNA synthesis and increased p53 protein levels after DNA damage (after XRT and actinomycin D and with caffeine and CHX treatment of irradiated cells). If wild-type p53 protein is a critical participant in the inhibition of replicative DNA synthesis after DNA damage, then DNA synthesis in cells with abnormal p53 proteins should be less inhibited after DNA damage than cells with wild-type *p53*. Therefore, as a first step in addressing the question of a functional linkage between increases of wild-type p53 protein levels and G_1 arrest after DNA damage, we compared the

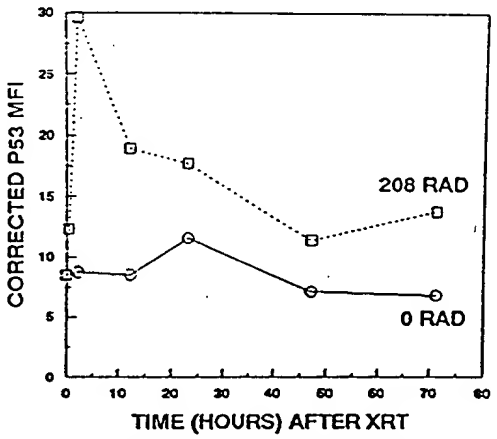


Fig. 3. Time course of changes in p53 protein levels in ML-1 cells exposed to XRT. The corrected *p53* MFI (the MFI of p421 antibody staining minus the MFI of staining with antibody plus inhibitory peptide, as described in "Materials and Methods") was measured in ML-1 cells at various times after exposure to 0 rads (—) or 208 rads (- - - -).

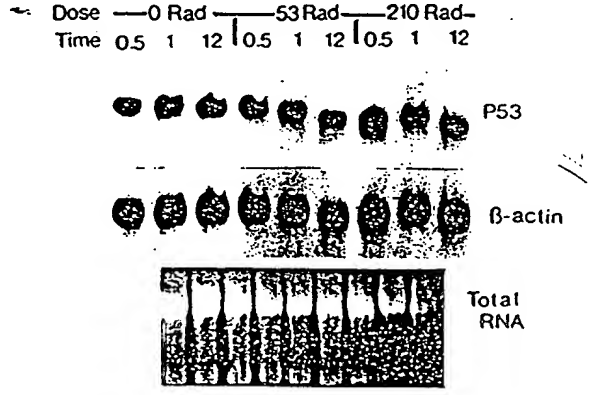


Fig. 4. Levels of *p53* mRNA in ML-1 cells exposed to XRT. Total cellular RNA was isolated from ML-1 cells at 0.5, 1, and 12 h after exposure to 0, 53, or 210 rads XRT. After electrophoresis and transfer, the RNA was hybridized with ^{32}P-labeled probes for *p53* or *β*-actin. Ethidium bromide staining of the agarose gel is shown as an additional control for the amounts of RNA loaded in each lane (20 *μg/lane*).

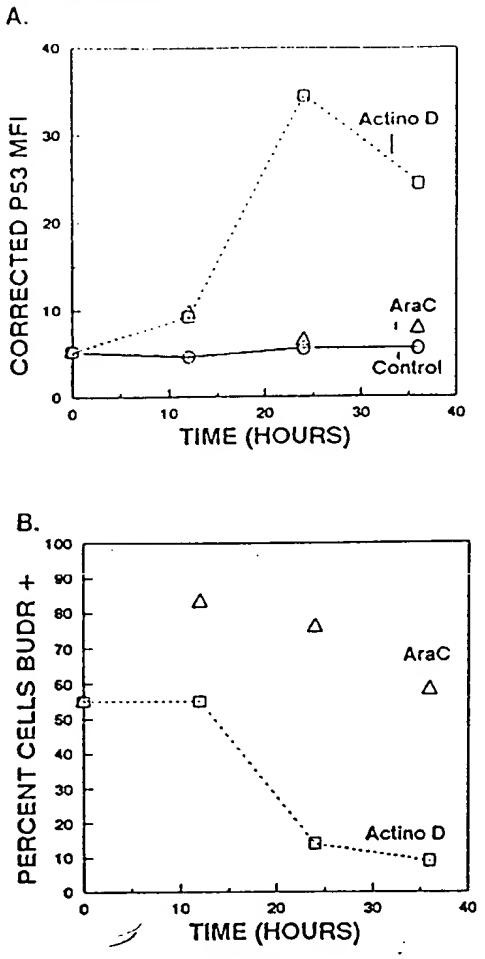


Fig. 5. Alterations in p53 protein levels and DNA synthesis in ML-1 cells exposed to actinomycin D or ara-C. *A*, levels of p53 protein (assessed as in Fig. 3) at various times during continuous exposure to 0.45 nM actinomycin D (Actino D; - - - -), 50 nM ara-C (· · ·), or no cytotoxic agent (—). *B*, percentage of cells in S-phase assessed by flow cytometry after a 4-h pulse of BrdUrd *(BUDR)* incorporation at various times during continous exposure to the same doses of actinomycin D (- - - -) and ara-C (· · · ·) noted in *A*.

changes in cell cycle progression after DNA damage in cells with altered *p53* genes to cells with normal *p53* genes.

Exons 5 through 9 of the *p53* genes from the myeloid leukemia cell lines, ML-1 and KG-1a, and the lymphoid leukemia cell lines, Raji and RPMI 8402, were amplified, subcloned, and sequenced as described previously (32). No mutations were found in ML-1 cells. KG-1a cells were found to have a mutation at codon 225, resulting in a substitution of isoleucine for valine on one allele, and loss of the other allele. Raji cells were found to have a wild-type *p53* allele and an allele with a G to A transition at codon 213 resulting in a glutamine for arginine substitution. RPMI 8402 cells had one wild-type *p53* allele and one allele with a C to T transition at codon 273 resulting in a cysteine for arginine substitution. In agreement with previous reports (40), HL-60 myeloid leukemia cells did not have an intact *p53* gene on Southern blot analysis (data not shown). Our recent characterizations of *p53* expression in these cell lines had revealed that Raji and RPMI 8402 cells express high p53 protein levels, as seen in other cell lines with *p53* gene mutations. ML-1 cells, on the other hand, had low levels, whereas KG-1a and HL-60 cells lacked both *p53* mRNA and protein expression (22). (It is not yet clear why the *p53* mutation in KG-1a cells results in a virtual absence of *p53* mRNA expression.) Thus, cells with mutant *p53* genes [either express-

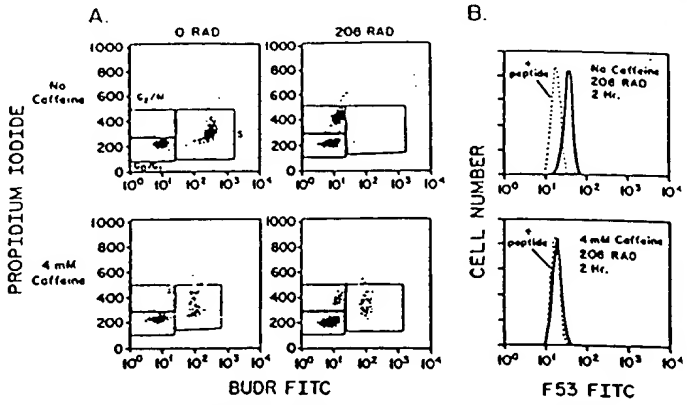
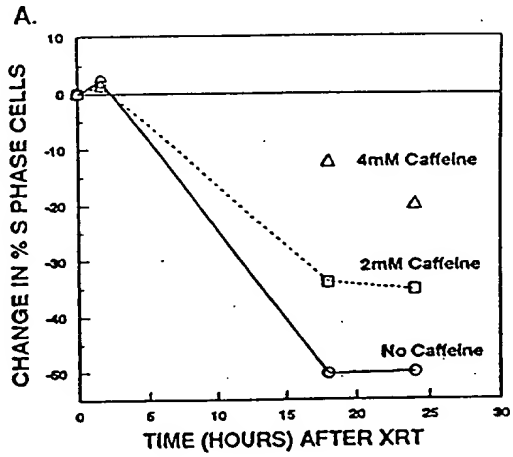


Fig. 6. Effects of caffeine on the alterations in cell cycle progression and p53 protein expression in ML-1 cells exposed to XRT. *A*, ML-1 cells, without (*top panels*) or with (*bottom panels*) 4 mM caffeine (added 30 min before XRT), were exposed to 0 (*left panels*) or 208 (*right panels*) rad. After 18 h, the cells were pulsed for 4 h with BrdUrd *(BUDR)* and the cell cycle populations were analyzed by BrdUrd/propidium iodide staining as described in Fig. 1*B*. *B*, ML-1 cells were analyzed for p53 protein expression (as described in Fig. 2*A*) 2 h after exposure to 208 rad in the absence (*top panel*) or presence (*bottom panel*) of 4 mM caffeine (added 30 min before XRT).



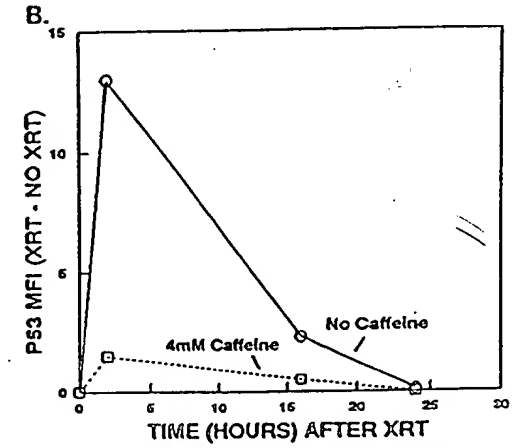


Fig. 7. Time course of the effects of caffeine on DNA synthesis and p53 protein levels in ML-1 cells exposed to XRT. ML-1 cells were exposed to 208 rad in the presence of 0, 2, or 4 mM caffeine and then analyzed for DNA synthesis (BrdUrd incorporation) and for p53 protein expression at various times. *A*, *points*, change in the percentage of cells in S-phase in irradiated compared with unirradiated cells at each time for each dose of caffeine. *B*, *points*, difference between the corrected *p53* MFI (as described in Fig. 3) in irradiated and unirradiated cells at each time for 0 and 4 mM caffeine.

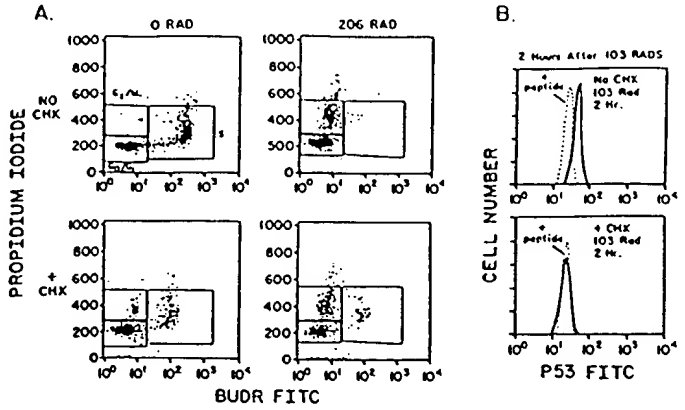


Fig. 8. Effects of cycloheximide on the changes in DNA synthesis and p53 protein levels in ML-1 cells after XRT. *A*, cell cycle populations characterized by BrdUrd (BUDR)/propidium iodide staining 15 h after exposure to 0 rad (*left panels*) or 206 rad (*right panels*) without (*top panels*) or with (*bottom panels*) exposure to 10 μM cycloheximide, which was added 10 min before XRT and washed out 5 h later. *B*, flow cytometric analysis of p53 protein expression (as described above) 2 h after exposure to 103 rad without (*top panel*) or with (*bottom panel*) 10 M cycloheximide added 10 min before irradiation. *FITC*, fluorescein isothiocyanate.

ing p53 protein (Raji and RPMI 8402 cells) or not expressing it (KG-1a)], as well as cells with no intact *p53* genes (HL-60) were available for comparisons to cells with no "hot-spot" *p53* mutations (normal bone marrow myeloid progenitors and ML-1 cells) in studies of the role of p53 protein in the cellular response to DNA damage (Table 1).

If these cells with abnormal or no *p53* genes exhibited arrests of DNA synthesis after DNA damage similar to ML-1 and bone marrow cells, then a significant role for normal p53 protein in this process would effectively be ruled out. However, in contrast to ML-1 cells and normal human bone marrow myeloid progenitor cells, the myeloid leukemia cell lines, HL-60 and KG-1a, and the lymphoid leukemia cell lines, Raji and RPMI 8402, all with altered *p53* genes, continued to progress through S-phase after γ-irradiation (Fig. 9*A*; data not shown for bone marrow and RPMI 8402 cells). Although all of the cell types exhibited an increase in the percentage of cells in G_2 after exposure to γ-irradiation (Fig. 9*A*), only ML-1 cells and normal bone marrow progenitor cells exhibited a G_1 arrest (Fig. 9*B*; data not shown for RPMI 8402). These observations suggest that the wild-type *p53* gene participates in the inhibition of replicative DNA synthesis via a G_1 arrest after DNA damage, but probably does not significantly contribute to the G_2 arrest.

DISCUSSION

Arrest of replicative DNA synthesis after DNA damage is thought to occur to provide ample time for the cell to repair DNA lesions before S-phase (G_1 arrest) and/or mitosis (G_2 arrest). The mechanisms underlying this arrest are poorly understood (6). In this study, we have begun to characterize the cellular mechanisms responsible for this arrest of DNA synthesis in mammalian cells. The *p53* gene is thought to function as an inhibitor of cellular replication, thus allowing the unregulated growth of the tumor cells when it is abnormal (9, 10, 41). Our data confirm that the inhibition of DNA synthesis is an active physiological response to DNA damage in mammalian cells (since it is blocked by both caffeine and CHX) and suggest that one function of the p53 protein is to participate in the cellular response to DNA damage, perhaps by transient inhibition of new replicative DNA synthesis.

The observations that only cells with normal *p53* genes exhibit a rapid increase in the levels of wild-type p53 protein after DNA damage, as well as a temporally correlated G_1 arrest, suggest that wild-type p53 protein is an active participant in the G_1 arrest that follows DNA damage. Interestingly, it appears that either loss of expression of wild-type p53 (a recessive mechanism) or overexpression of a mutant *p53* gene (a dominant mechanism) can result in this abnormal cell cycle response to XRT. In contrast to the G_1 arrest, however, all cell types, regardless of *p53* gene status, exhibited a G_2 arrest after XRT. Consistent with the lack of inhibition by CHX of the G_2 arrest in yeast after XRT (39), brief exposures to CHX blocked the p53 protein induction and inhibited the G_1 arrest, but did not block the G_2 arrest. A role for p53 protein in this G_1 arrest is consistent with other experiments demonstrating that transfection of wild-type *p53* genes into various tumor cell lines induces a G_1 arrest (10–13). In addition, our observation that cells in S-phase continue to progress through to G_2 after XRT (Fig. 1*B*) is also consistent with a role for *p53* in this process, since S-phase cells are immune to the *p53* negative regulation of growth until they enter the next G_1 period (13).

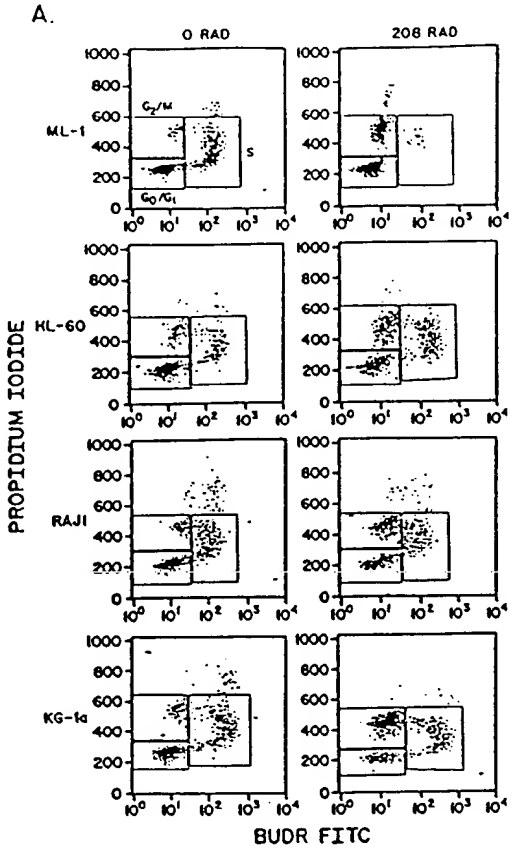
Our results in hematopoietic cells are consistent with, and extend, an earlier observation that treatment of nontransformed mouse fibroblasts with UV irradiation or a UV-mimetic chemical carcinogen caused a rapid increase in the amount of p53 protein (23). Interestingly, as we observed with the *p53* mutant-overexpressing Raji cells, no increase in p53 protein levels was seen in SV40-transformed mouse fibroblasts (which overexpress p53 protein due to binding of T antigen) after DNA damage (23). Thus, p53 protein levels increase after DNA damage in different tissue types with wild-type *p53* genes (hematopoietic cells and fibroblasts) and following different types of DNA damaging agents [XRT, actinomycin D, UV, and UV-mimetics (the latter 2 from Ref. 23)]. In contrast, exposure of cells to cytosine arabinoside, a cytotoxic agent that neither significantly damages DNA nor induces a G_1 arrest, did not cause significant changes in p53 protein levels.

Since there are no detectable changes in the levels of *p53* mRNA after XRT, and since p53 protein levels increase despite

Table 1 *Status of P53 gene, protein, and XRT response in selected cell types*

Cell	Lineage	P53 gene[c]	Mutant codon	A.A. Δ[c]	P53 protein[a]	G_1 arrest[b]
NBMP[c]	Myeloid	WT	–	–	+/–	+
ML-1	Myeloid	WT	–	–	+	+
KG-1a	Myeloid	M	225	VAL→ILE	–	–
HL-60	Myeloid	A	–	–	–	–
Raji	Lymphoid	M/WT	213	ARG→GLN	+++	–
RPMI8402	Lymphoid	M/WT	273	ARG→CYS	+++	–

[a] Relative levels of p53 protein as described here and in Kastan *et al.*, (22); +++, high levels; +, low levels; –, no detectable protein; +/–, proliferative NBMP cells normally have no detectable p53 protein, but express low levels after XRT.
[b] G_1 arrest after XRT.
[c] NBMP, normal bone marrow progenitor cells rapidly growing in liquid culture (only myeloid progenitor cells grow under these conditions); M, mutant p53 gene; WT, wild-type p53 gene; A, absent p53 gene; A.A.Δ, amino acid change.



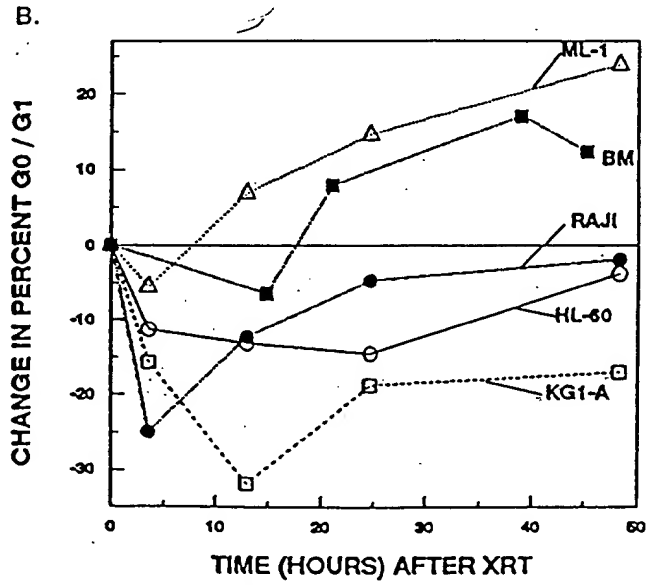


Fig. 9. Cell cycle changes in various cell types after exposure to XRT. *A*, cell cycle populations in ML-1, HL-60, Raji, and KG-1a cells 13 h after exposure to 0 rad (*left panels*) or 208 rad (*right panels*) were characterized by BrdUrd *(BUDR)*/ propidium iodide staining as described in Fig. 1*B*. *B*, quantitation of the changes in the percentage of cells in G_0/G_1 (assessed as in *A*) in ML-1, HL-60, Raji, KG-1a, and normal human bone marrow myeloid progenitor cells (*BM;* isolated and grown in culture as described in "Materials and Methods") at various times after exposure to 208 rads. *FITC*, fluorescein isothiocyanate.

the use of actinomycin D at doses that can significantly inhibit RNA synthesis (36), this increase is likely brought about by a posttranscriptional mechanism. Again, this is consistent with the observation by Maltzman and Czyzyk (23), that the increase in p53 protein in murine fibroblasts after UV damage is due to an increased half-life of the protein. Since we find that the increase in p53 protein is reversibly inhibited by CHX (added before XRT), continued synthesis of p53 protein may be required. However, the synthesis of another gene product involved in the induction or stabilization of p53 protein could also be important.

A posttranscriptional mechanism for the increase in p53 protein after DNA damage would avoid the necessity of transcribing new RNA utilizing a potentially damaged DNA template. If increases in the half-life of p53 protein are occurring after DNA damage, such increases could result from changes in phosphorylation, binding to other proteins, or oligomerization of *p53*, all of which have been reported to occur (19, 42–45). If phosphorylation changes are involved in this process, then $p34^{cdc2}$ may be a logical kinase to investigate, since: (*a*) *p53* has been found to be associated with $p34^{cdc2}$ kinase in transformed cells (46); (*b*) *p53* is a substrate for $p34^{cdc2}$ *in vitro* (44); and (*c*) γ-irradiation rapidly inhibits $p34^{cdc2}$ activity (47). Alternatively, our observations that caffeine exposure inhibits both the arrest of replicative DNA synthesis and the increase in p53 protein levels not only further implicates p53 protein in this process, but also suggests the possible involvement of a cAMP- or cGMP-dependent process (such as a cyclic nucleotide-dependent kinase) in the up-regulation of p53 protein after DNA damage.

Cells with mutant *p53* genes continue to go through S-phase after DNA damage; thus, use of a damaged template for replicative synthesis in these cells might lead to significant mutations, and possibly to genomic instability, in their daughter cells. For example, mutations of the *RAD9* gene in yeast permit cells with unrepaired DNA damage to proceed through the cell cycle, rather than arresting in G_2, and DNA damage of these cells results in increased numbers of cells with chromosomal aberrations (6). Thus, if the cessation of replicative DNA synthesis after DNA damage is a critical cellular response for minimizing the chance of cellular transformation, then our observations suggest a new mechanism for how the loss of wild-type *p53* might contribute to tumorigenesis and to the progressive genetic changes, chromosomal abnormalities, and aneuploidy commonly observed in tumor cells (9, 48). The high frequency of *p53* mutations in a wide variety of tumors, many of which have been linked to carcinogen exposures, underscores the potential significance of this type of mechanism in the development and progression of human cancers.

Patients with the familial cancer-prone syndrome, Li-Fraumeni, have germ line mutations in the *p53* gene (49, 50). Consistent with the possible function of wild-type p53 protein discussed above and the associated physiological consequences of the presence of abnormal *p53* expression and/or function, fibroblasts from patients with Li-Fraumeni syndrome may have an increased incidence of developing aneuploidy in tissue culture compared with fibroblasts from normal individuals (51). In addition, epidemiological data have suggested that an increased susceptibility to carcinogenesis by exposure to environmental carcinogens, such as ionizing radiation and tobacco smoke, may contribute to the array of tumors seen in this syndrome (52, 53). Thus, this type of role for p53 protein in the response to DNA damage may also help to explain why these patients can develop a wide variety of tumor types (this pathway is not tissue-specific) and have a median age of over 30 years for the development of their first tumor.

In conclusion, these experiments demonstrate that p53 pro-

tein levels increase after DNA damage, probably via a posttranscriptional mechanism, and may contribute to a transient inhibition of replicative DNA synthesis. Further characterization of this process should provide additional insights into physiological mechanisms that contribute to radiation- and chemically induced carcinogenesis. It should also provide information about the function of wild-type p53 protein in normal cells and the mechanism(s) whereby alterations in *p53* contribute to tumorigenesis.

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Advances in Brief

Loss of a *p53*-associated G1 Checkpoint Does Not Decrease Cell Survival following DNA Damage[1]

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Abstract

Cell cycle checkpoints regulate progression through the cell cycle. In yeast, loss of the G_2 checkpoint by mutation of the *rad9* gene results in increased genetic instability as well as increased sensitivity to ionizing radiation. In contrast, comparing clonogenic survival of cells which are isogeneic except for *p53* functional status, we find that loss of a G_1 checkpoint in mammalian cells is not associated with increased sensitivity to the lethal effects of ionizing radiation or a topoisomerase I inhibitor, camptothecin. These results indicate that increased sensitivity to DNA-damaging agents is not necessarily a defining feature of a mammalian cell cycle checkpoint. Furthermore, in light of a recent link of *p53* function to radiation-induced apoptosis in hematopoietic cells, these observations suggest that p53-dependent apoptosis is a cell type-specific phenomenon and thus predict that the biological consequences of loss of *p53* function will be cell type specific.

Introduction

Damage to the DNA of proliferating cells causes alterations in progression through the cell cycle (*e.g.*, Refs. 1 and 2). Hartwell and Weinert (3–5) suggested that the definition of a cell cycle checkpoint included that it was: (*a*) nonessential; (*b*) inhibitory; and (*c*) transduced a signal. The role of *p53* in causing an arrest of mammalian cells in the G_1 phase of the cell cycle following IR[3] fulfilled such criteria (6–8). Although the physiological ramifications of loss of the G_2 arrest checkpoint in yeast by mutation of the *rad9* gene included both decreased cell survival and increased genetic instability following DNA damage, it was not obvious whether loss of the *p53*-dependent mammalian G_1 checkpoint would have similar biological consequences. Loss of *p53* function has been associated with an increased tendency for gene amplification and for the development of aneuploidy (9–11), thus suggesting that increased genetic instability is a consequence of loss of this checkpoint function. In this paper we have now addressed the question of whether loss of this *p53*-dependent checkpoint influences sensitivity to the cytotoxic effects of ionizing radiation or a topoisomerase I poison.

Materials and Methods

Cells. SW480 colorectal carcinoma cells, parental RKO colorectal carcinoma cells, which have wild-type *p53* alleles (7, 12), and RKO cells transfected with either control vectors, vectors containing an overexpressed mutant *p53* transgene, or an overexpressed HPV-16 *E6* gene were grown and maintained as previously described (7, 8, 13). Normal embryonic fibroblasts from mice in which zero (p45.41C), one (p45.41B), or two (p45.41A and p45.41E) *p53* alleles had been disrupted were maintained as previously described (8).

Clonogenic Assays. Cells from a specific cell line in log phase growth were harvested and inoculated into several 25-cm^2 culture flasks with 5.0 ml media without G418. The flasks were incubated 2–3 days at 37°C until they approached 60–80% confluence, when they were exposed to a single dose of IR at prescribed doses [0 to 1600 cGy at ~100 cGy/min rads utilizing a ^{137}Cs source as previously described (7, 8)]. The cultures were incubated overnight and the following day cells from each flask were trypsin harvested, washed, and diluted to at least two different clonal densities prior to inoculation into 6-well plates. Varying numbers of cells were inoculated into 6-well plates, such that each IR dose and inoculum size was represented in triplicate. Three ml of media without G418 were added to each well and the plates were incubated for 4 days at 37°C. (Cell line p45.41C required 6 days growth for colony maturation.)

Prior to counting colonies, medium was decanted from each plate and wells were fixed in methanol and stained with fast green. A low magnification microscope was used to identify colonies, which were counted only if they numbered more than 30 cells each. The mean ± SE for colony counts appear in the figures.

Results

Comparisons of the clonogenic survival curves of two colorectal carcinoma cell lines, SW480 [which contains mutant *p53* genes and exhibits no IR-inducible G_1 checkpoint (7)] and RKO cells [which have wild-type *p53* alleles and a normal G_1 checkpoint (7)] revealed no significant differences in radiosensitivity (Fig. 1*A*). This demonstrated that loss of *p53* function is not a sole determinant of radiosensitivity. However, since other genetic differences between these two lines might also influence radiosensitivity, we extended our studies to comparisons of cells which were isogeneic except for the functional status of the *p53* gene product and which had been previously characterized in terms of the G_1 checkpoint status (7, 8, 13).

RKO cells stably transfected with an overexpressed mutant *p53* gene lost the G_1 arrest (7), but exhibited no alterations in radiosensitivity relative to parental RKO cells (Fig. 1*A*) or control transfectants (data not shown). Similarly, several different clonal isolates of RKO cells transfected with human papillomavirus *E6*, a gene whose protein product binds avidly to *p53* protein and thereby inactivates the G_1 checkpoint (13, 14), exhibited no demonstrable alterations in radiosensitivity compared to parental RKO cells or control transfectants (Fig. 1*B*). In addition to the lack of differences in colony numbers, no obvious differences in colony sizes were noted as a function of *p53* functional status.

Similar experiments were performed utilizing embryonic fibroblasts derived from mice in which zero, one, or two copies of the wild-type *p53* genes were disrupted by homologous recombination (8, 9). The fibroblast lines with *p53* gene deletions have abnormalities of the G_1 checkpoint, while the line with two wild-type *p53* alleles has normal checkpoint function (8). Significant differences in clonogenic survival were not observed in these otherwise isogeneic normal cells (Fig. 2). Although the ploidy of these cells was not assessed at the exact time these studies were done, these fibroblast cultures were at very early passage (<5), a time when the homozygous *p53* knockout cells still exhibit a relatively normal chromosomal complement (9).

In order to demonstrate that the radiosensitivity of the RKO cells could actually be altered by some manipulation, the cells were exposed to caffeine prior to irradiation. Caffeine treatment, which inter-

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: IR, ionizing radiation; A-T, ataxia-telangiectasia.

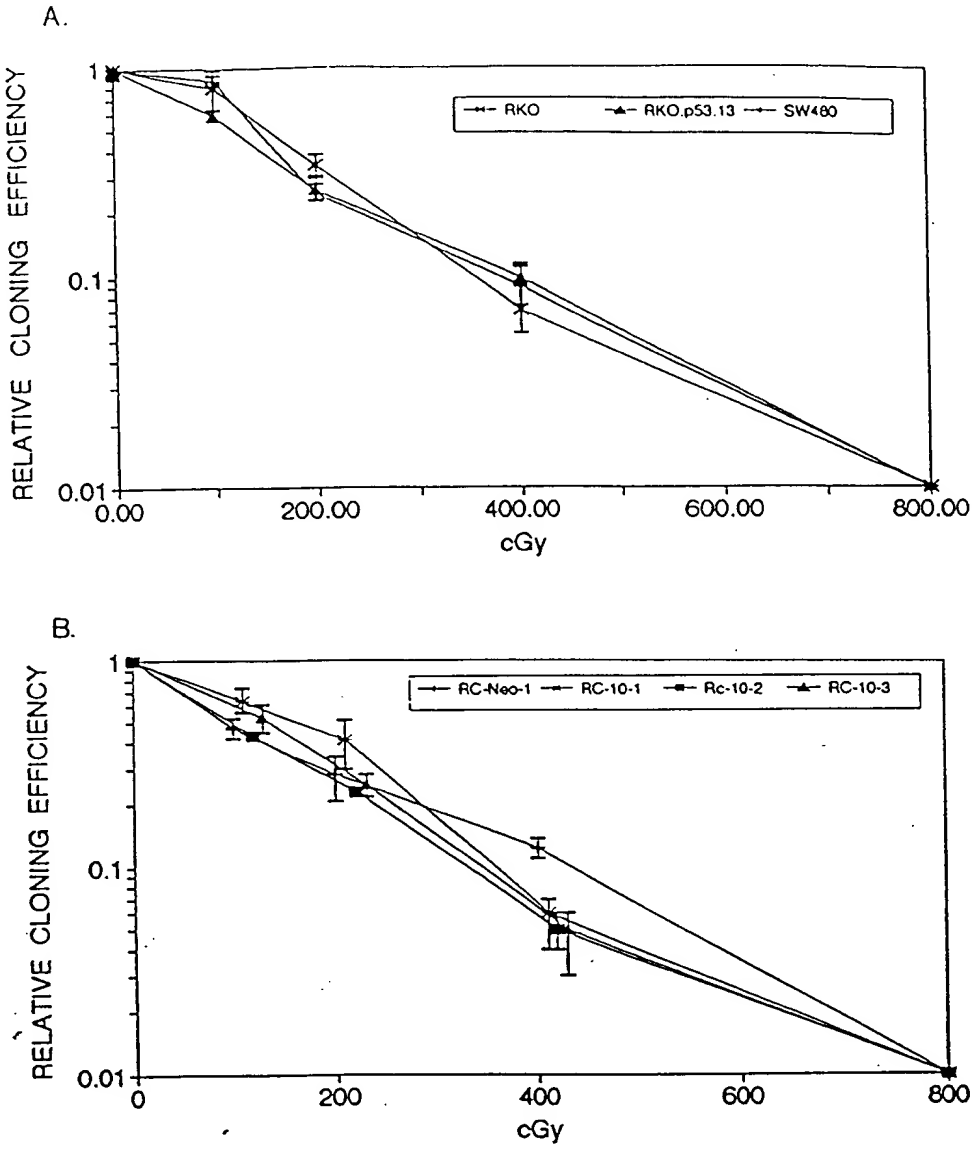

Fig. 1. *A*, clonogenic survival of colorectal carcinoma cells with varying or manipulated *p53* functional status. Survival as a function of IR dose for SW480 cells (endogenous mutant *p53*), RKO (endogenous wild-type *p53*), and RKO.p53.13 (RKO clone with transfected mutant *p53*). *B*, survival of RKO cells stably transfected with vector alone (RC-Neo-1) or three separate clonal isolates transfected with HPV-16 *E6* (RC-10-1; RC-10-2; RC-10-3). All doses for a given cell line were processed simultaneously. *Points*, mean of triplicate colony counts; *bars*, SD.

feres with both the G_1 and G_2 arrests (1, 6, 15) and increases the cytotoxicity of IR (16), increased the radiosensitivity of RKO cells as expected (Fig. 3). Since our data suggest that loss of the G_1 arrest does not lead to increased radiosensitivity, the results with caffeine implicate loss of the G_2 arrest as one potential mediator of radiosensitivity. This would be consistent with the yeast model (4) and data suggesting that G_2 is a particularly radiosensitive stage of the cell cycle (17).

Inhibitors of topoisomerases I and II lead to DNA strand breakage (18) and cause increases in the levels of wild-type p53 protein similar to those seen following IR (19).[4] Since such agents are commonly used therapeutically, we also evaluated clonogenic survival after exposure to the topoisomerase I inhibitor camptothecin. Again, no significant *p53*-dependent changes in sensitivity to the cytotoxic agent were observed (Fig. 4). Similar results were found in a prostate carcinoma cell line (LNCap) following stable transfection with mutant *p53* and exposure to camptothecin (data not shown).

Discussion

These data demonstrate no direct influence of *p53* gene status or G_1 checkpoint status on the sensitivity of these cells to the lethal effects of IR. At first glance, this might appear to be somewhat contradictory to the recent observations that thymocytes from mice in which the *p53* alleles had been disrupted (20, 21) and hematopoietic cells from mice expressing a mutant *p53* allele (22) appear to be relatively radioresistant. The mechanism of the radioresistance in the thymocytes appeared to be loss of a signal which induces apoptosis following IR (20, 21).

These results are not necessarily contradictory. First, both sets of observations actually agree that loss of *p53* and G_1 checkpoint function does not lead to increased radiosensitivity, which had been our original bias because of the consequences of loss of *rad9* and G_2 checkpoint function in yeast (3–5). Second, IR does not induce apoptosis in all cell types; lymphoid cells are particularly sensitive to IR and are known to undergo apoptosis following exposure to IR and many other agents (23). The most plausible explanation for these differences is that there are cell type-specific differences in the response to irradiation: some cell types may undergo an IR-induced G_1 arrest (*e.g.*, fibroblasts), while an apoptosis pathway is set in motion by IR in other cell types (Fig. 5). It is plausible that the proximal arm of both pathways (up to IR induction of p53 protein levels) is shared by all cells and then some cell types use this p53 signal to initiate apoptosis, while others initiate a G_1 arrest signal. For cells which lose

[4] W.G. Nelson and M.B. Kastan, manuscript in preparation.

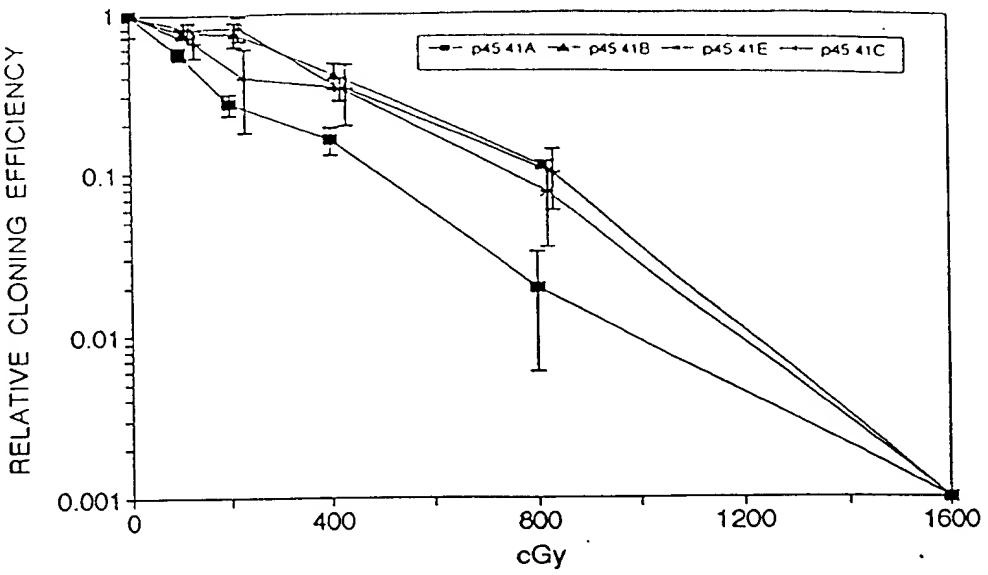

Fig. 2. Clonogenic survival of fibroblasts from *p53* "knockout" mice with zero deletions of *p53* (p45.41C), single deletion (p45.41B), or two deletions (p45.41A and p45.41E). Subline p45.41C grows more slowly than the other lines, and incubation for 6 days was required prior to fixation and counting of colonies.

p53 function, such a scenario would result in radioresistance if the cell normally utilized the apoptosis pathway; in contrast, in cells utilizing the G_1 arrest pathway, radiosensitivity would only be affected if that were a consequence of loss of this checkpoint. Our data suggest that loss of the G_1 checkpoint alone does not lead to significant increases or decreases in radiosensitivity.

It is also worth noting that different end points of survival are used here compared to the experiments done with irradiated thymocytes (20, 21). Since clonogenic survival could not be assessed in the thymocytes as we did here, cell death was evaluated by membrane permeability assays. This permeability was assessed within 24 h, while clonogenic survival here reflected at least 4 days of growth after irradiation and a minimum colony size of 30 cells. However, it is unlikely that these differences in results reflect assay differences because colony-forming assays done with bone marrow progenitor cells expressing a mutant *p53* transgene also exhibited a relatively radioresistant phenotype (22). Therefore, the most likely explanation remains that cells of hematopoietic lineage utilize induction of *p53* to undergo apoptosis after IR, while fibroblasts and colorectal tumor cells do not. This scenario is compatible with the observations that overexpression of wild-type p53 protein in a murine hematopoietic cell line utilizing a temperature-sensitive *p53* gene product resulted in apoptosis (24), while only growth arrest has been noted in other cell types (such as fibroblasts) expressing this gene product in the wild-type conformation (25).

Skin fibroblasts from members of a family with the Li-Fraumeni syndrome, which is usually associated with germ line mutations of the *p53* gene (26, 27), have been reported to be relatively radioresistant (28). However, these fibroblasts also were found to have increased expression of the c-*myc* oncogene and alterations in c-*raf* activation, both of which have been linked to alterations in radioresistance (29, 30). Consistent with our findings, the radiosensitivity of a small number of cell lines derived from human head and neck squamous carcinomas had no consistent relationship to *p53* gene status (31). Our experiments extend such preliminary findings by utilizing cell types which are isogeneic except for *p53* functional status, and therefore are not susceptible to artifacts induced by the activation of other gene products that might influence radiosensitivity.

Our results are also in agreement with studies using hybrid cells produced by the fusion of ataxia-telangiectasia cells with HeLa cells [which express HPV-18 E6 which abrogates p53 function (32)] and studies fusing normal and A-T cells (33, 34), all of which suggested that the radiosensitivity of ataxia-telangiectasia and "radioresistant DNA synthesis" are under separate genetic controls. Since optimal

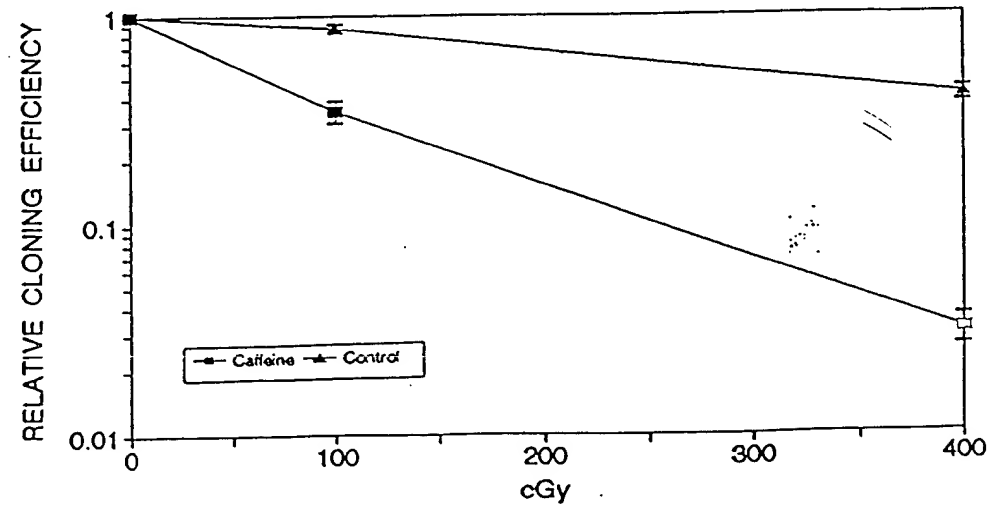

Fig. 3. Clonogenic survival of RKO cells in the presence of caffeine. Caffeine dissolved in methanol was added to treated flasks 20 min prior to irradiation, to final concentrations of 2.0 mM caffeine and 1% methanol (v/v). Control cells received 1% methanol (v/v) alone, which resulted in lower clonogenic survival at 0 cGy than seen in Fig. 1 and accounted for the higher relative survival seen in irradiated specimens here.

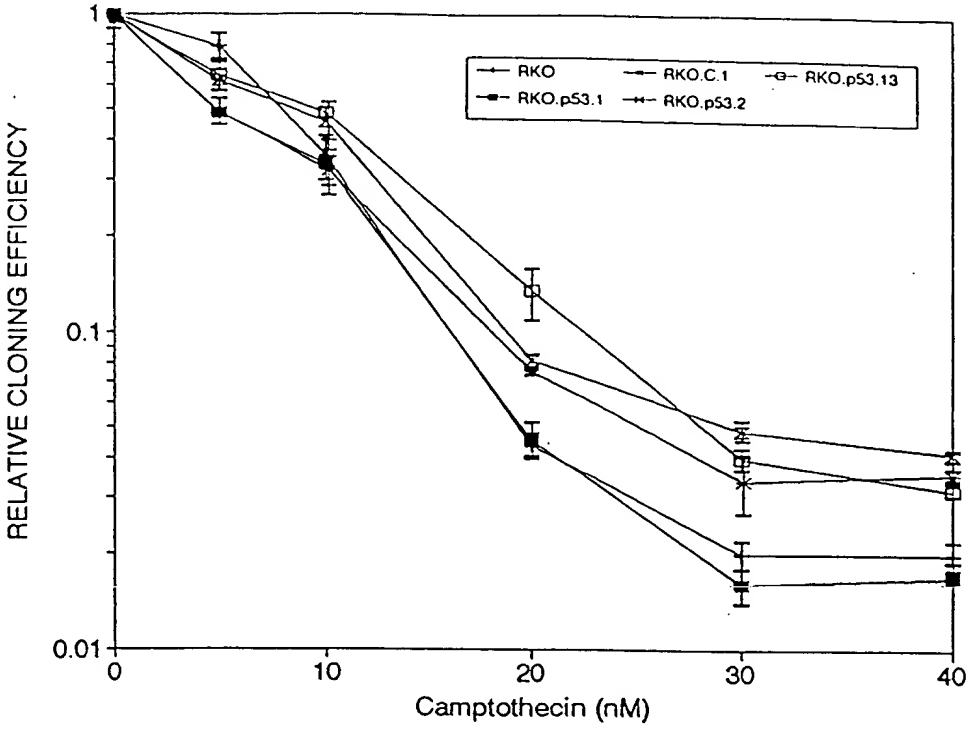

Fig. 4. Clonogenic survival of RKO and transfectant cell lines after exposure to camptothecin. RKO, control transfectant (RKO.C.1), transfected mutant *p53* (RKO.p53.1; RKO.p53.2; RKO.p53.13). Cells were plated in individual wells at clonal density and camptothecin was added to the specified concentrations. After 24 h of exposure to CPT, the wells were washed 3 times with complete media and then incubated for 96 h prior to fixation, staining, and counting.

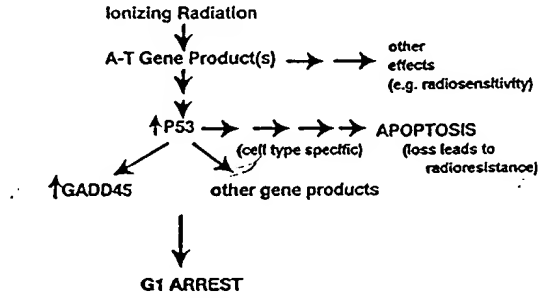

Fig. 5. Proposed scenario of events resulting from cellular irradiation. Following exposure of cells to ionizing radiation, p53 protein levels increase in a posttranscriptional mechanism; optimal p53 induction requires normal function of the genes defective in A-T. Abnormal function of the A-T gene(s) has other biological consequences independent of its effects on p53, such as leading to increased radiosensitivity and abnormalities in both S phase and G_2 arrests. Induction of p53 by IR leads to a G_1 arrest in certain cell types (*e.g.*, fibroblasts) and to apoptosis in other cell types (*e.g.*, hematopoietic cells). Loss of p53 function would lead to radioresistance in cell types utilizing the apoptosis part of the pathway.

induction of p53 protein levels by IR appears to require normal A-T gene products (8), but since we do not observe decreased cell survival with loss of p53 function, it is likely that loss of A-T gene function has many biological consequences, only one of which is suboptimal induction of p53 after IR. This suggests that the reason A-T cells are radiosensitive is not due to suboptimal induction of p53, but because of some other response dependent on the A-T gene product(s) (Fig. 5).

These results indicate that although the cell cycle checkpoint in G_1 can be impaired through mutation of *p53* or by other mechanisms, that loss the G_1 checkpoint *per se* does not influence radiosensitivity or sensitivity to camptothecin. Since caffeine treatment enhances radiosensitivity and since caffeine abolishes both the G_1 and G_2 checkpoints, the loss of the G_2 checkpoint may be a more important determinant of radiosensitivity. This hypothesis is consistent with the findings in ataxia telangiectasia, where defects in both the G_1 and G_2 arrests can be present, and in the yeast *rad9* mutants which lack a normal G_2 checkpoint: both A-T cells and *rad9* mutants are radiosensitive. Further study will be required to clarify the relationship of the G_2 checkpoint to radiosensitivity. Combining current observations in yeast and mammalian systems, increased genetic instability may be a ramification of loss of all (or most) cell cycle checkpoints, while cell survival does not appear to be affected by all checkpoints.

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PTO/SB/ 26 (10-96)

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PRIOR PATENT	Docket No. (Optional) INRP:050/HYL

In re Application of: Jack A. Roth, Toshiyoshi Fujiwara, Elizabeth A. Grimm, Tapas Mukhopadhyay, Wei-Wei Zhang, and Laurie B. Owen-Schaub
Application No.: 08/918,407
Filed: August 26, 1997
For: METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND p53

Petitioner, Board of Regents, The University of Texas , is the owner of 100 percent interest in the instant application. Petitioner hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. §§ 154 to 156 and 173, as presently shortened by any terminal disclaimer, of prior Patent No. 5,747,469. Petitioner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, petitioner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. §§ 154 to 156 and 173 of the priorpatent, as presently shortened by any terminal disclaimer, in the event that it later: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 C.F.R. § 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Pursuant to 37 C.F.R. § 3.73, the undersigned has reviewed the evidentiary documents, specifically, the Assignment of the instant application to Board of Regents, The University of Texas System, which has been recorded at Reel 7102/Frame 0696, and certifies that, to the best of his or her knowledge and belief, title of the instant application is in the name of Assignee, Board of Regents, The University of Texas System.

1. X For submissions on behalf of an organization (*e.g.*, corporation, partnership, university, government agency, etc.), the undersigned (whose title is supplied below) is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ___ The undersigned is an attorney of record.

Board of Regents, The University of Texas System

10/24/00
Date

By: Cullen M. Godfrey
Vice Chancellor and General Counsel

⊗ Terminal disclaimer fee under 37 C.F.R. § 1.20(d) included.

⊗ PTO suggested wording for terminal disclaimer was:

☐ unchanged. ☒ changed (if changed, an explanation should be supplied).

Paragraph in bold added for compliance with 37 C.F.R. § 3.73

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